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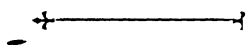
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CONTENTS

No. 1, JANUARY 1934

	PAGE
Recent studies in mass physiology. By W. C. ALLEE	I
Die Sichtbarkeit ultraviolettten Lichtes. Von E. MERKER	49
Chemical heterogony and the ground-plan of animal growth. By JOSEPH NEEDHAM	79
Esquisse d'une histophysiologie comparée du rein des Vertébrés. Par P. GÉRARD et R. CORDIER	110
The interpretation of phyllotaxis. By MARY SNOW and R. SNOW	132

No. 2, APRIL 1934

Cyclic reproduction, sex determination and depression in the Cladocera. By KAJ BERG	139
The haemocyanins. By ALFRED C. REDFIELD	175
The bird fauna of the Galapagos Islands in relation to species formation. By HARRY S. SWARTH	213
The proteolytic enzymes of micro-organisms. By R. B. HAINES	235

No. 3, JULY 1934

The symplasmic state of the tissues of the animal body. By F. K. STUDNÍČKA	263
The endocrine glands and calcium metabolism. By H. ZWARENSTEIN	299
The bacteriophages. By F. M. BURNET	332
Factors affecting the pathogenicity of cereal foot-rot fungi. By S. D. GARRETT	351

No. 4, OCTOBER 1934

The digestion of wood by insects and the supposed role of micro-organisms. By K. MANSOUR and J. J. MANSOUR-BEK	363
The derivation of the nitrogen of crop plants, with special reference to associated growth. By HUGH NICOL	383
The experimental production of mutations. By N. W. TIMOFÉEFF-RESSOVSKY	411
Concerning the evolution of the Cephalopoda. By O. H. SCHINDEWOLF	458
Genes and inductors of sex differentiation in amphibians. By EMIL WITSCHI	460

INDEX OF AUTHORS

	PAGE
ALLEE, W. C. Recent studies in mass physiology	I
BERG, KAJ. Cyclic reproduction, sex determination and depression in the Cladocera	139
BURNET, F. M. The bacteriophages	332
GARRETT, S. D. Factors affecting the pathogenicity of cereal foot-rot fungi	351
GÉRARD, P. et CORDIER, R. Esquisse d'une histophysiologie comparée du rein des Vertébrés	110
HAINES, R. B. The proteolytic enzymes of micro-organisms	235
MANSOUR, K. and MANSOUR-BEK, J. J. The digestion of wood by insects and the supposed rôle of micro-organisms	363
MERKER, E. Die Sichtbarkeit ultravioletten Lichtes	49
NEEDHAM, JOSEPH. Chemical heterogony and the ground-plan of animal growth	79
NICOL, HUGH. The derivation of the nitrogen of crop plants, with special reference to associated growth	383
REDFIELD, ALFRED C. The haemocyanins	175
SCHINDEWOLF, O. H. Concerning the evolution of the Cephalopoda	458
SNOW, MARY and SNOW, R. The interpretation of phyllotaxis	132
STUDNÍČKA, F. K. The symplasmic state of the tissues of the animal body	263
SWARTH, HARRY S. The bird fauna of the Galapagos Islands in relation to species formation	213
TIMOFÉEFF-RESSOVSKY, N. W. The experimental production of mutations	411
WITSCHI, EMIL. Genes and inductors of sex differentiation in amphibians	460
ZWARENSTEIN, H. The endocrine glands and calcium metabolism	299

RECENT STUDIES IN MASS PHYSIOLOGY

BY W. C. ALLEE.

(The University of Chicago.)

(Received February 24, 1933.)

(With Seven Text-figures.)

CONTENTS.

	PAGE
I. Introduction	1
II. Formation and integration of aggregations	2
III. Biologically conditioned media	4
IV. Mass effects on growth of individuals	5
V. Growth in tissue culture	7
VI. Mass stimulation of growth	8
VII. Mass effects on population	11
VIII. Effects of numbers present on oxygen consumption	19
IX. Mass protection from toxic conditions	21
X. Morphological effects	24
XI. Crowding and the phase theory of locusts	25
XII. The control of sex in Cladocera	28
XIII. Group influence on behaviour	34
XIV. Group organisation	39
XV. Summary	41
References	43

I. INTRODUCTION.

Mass physiology is a living, rapidly developing subject. Its most generalised form is to be found in the studies of ecological communities which fill many pages of current ecological journals. These are concerned both with the plant and the animal components of the communities and, happily, in an increasing number of cases, with the entire biota (Allee, 1932). The simplest form of these community studies finds its expression in modern plant sociology, which is concerned with such subjects as the organisation of the community with regard to various degrees of community influence of the constituent plants; the fidelity¹ of plants to a given community; their frequency of appearance and degree of gregariousness; the mutual relations between the community and its environment; and the whole of the complicated matter of the evolution of communities (Braun-Blanquet, 1932).

¹ This review is undertaken in connection with a research programme in animal aggregations which is supported in part by a grant from the Rockefeller Foundation to aid investigations in the biological sciences at the University of Chicago. I am indebted to many associated students for permission to include the results of their investigations in advance of publication, although in no case in advance of the preparation of their research report in final manuscript form. I am particularly indebted to Miss Gertrude Evans for aid in searching the literature.

In more specialised form, mass physiology is concerned with the study of the more intimate groupings which find their simplest expression among animals in the aggregations of many individuals of the same species in a limited space. The present review will be limited to a discussion of recent work in the narrower rather than in the wider field. As such it will serve as a supplement to my extended review of the subject which appeared in 1931 under the title of *Animal Aggregations, a Study in General Sociology*. While it may be necessary at times for the sake of clearness to outline previous developments in the subject, care will be taken to avoid repetition of subject-matter or of bibliographic references to work which has been adequately treated in that volume. In the main this review will be limited to a consideration of research reports which have appeared in the two years and more since that manuscript was closed; while some references will be made to papers which will appear in 1933, no systematic effort has been made to include work published in that year.

II. FORMATION AND INTEGRATION OF AGGREGATIONS.

There has been relatively little progress in the analysis of the methods of formation of animal groups in the period covered by this review. The recording of assemblies due to sex attraction has continued. Winged phasmid males collect about a caged wingless female, and similar aggregations occur in another phasmid species, both sexes of which are wingless (Taylor, 1931). Sex attraction in the giant saturnid moths has been analysed to some extent (Rau, 1929) to show that the expression of sex attraction is affected by a rhythmic periodicity which in turn is affected in part by light periodicity, even though the response of the males is due primarily to odour perception. This rhythmic periodicity in *Cecropia* can be changed to a considerable degree by simulating at high noon the light conditions characteristic of dawn.

Many of even the recent studies of the formation of aggregations do not attempt to go beyond the recognition of some sort of social instinct. Fraenkel (1929) reports that the South African locust, *Schistocerca gregaria*, collects because of an instinct for aggregation and shows group behaviour in part as a result of this instinct and in part as a result of the so-called instinct for imitation. Jones (1930, 1931) in his record of the sleeping aggregations of *Heliconius* in Florida, concludes that this butterfly aggregates at night as a result of a mechanism involving place-memory and a social instinct.

Despite the general reliance on the presence of some sort of a social instinct, some progress has been made in the study of schooling behaviour in fishes. Parr (1927) made a brave attempt to get behind the social instinct and concluded that the assumption of a simple automatic "attraction" toward a perceived companion formed a more satisfactory basis for an explanation of schooling. The behaviour of the schooling fishes, particularly of *Scombrus colias*, indicated that the automatic attraction is probably of a purely visual character. Accompanying this tendency to approach a companion, Parr found a mutual adjustment of behaviour which

infusions; with small volumes she obtained the same results as had the critics of Robertson.

Recently Di Tomo (1932) has attempted to repeat Petersen's work, using the same species of *Paramecium* and testing comparable volumes. She used, however, a sterile medium and obtained only negative results. She concludes that any substance given off by *Paramecium* not only fails to have an accelerating effect on division but produces rather a toxic effect, and that the induced toxicity is proportional to the initial numbers of animals present in the experimental cultures.

Exactly opposed results, in so far as they test the same question, are reported by Dimitrowa (1932). In this work an attempt was made to test the effect of excretion products on the rate of division of *P. caudatum*. Single animals were isolated into a medium composed of 12 drops of water and 4 drops of a rich bacterial culture. After 24 hours under favourable and reproducible conditions the progeny from such isolated animals were used in an experiment. Negative results were obtained when the paramecia were isolated into 0.5 c.c. of culture medium which contained from 1 to 12 drops of centrifuged culture medium in which from 10 to 100 paramecia had lived for the preceding 24 hours. When only one small drop of a much less contaminated culture was introduced, the paramecia isolated into this slightly *Paramecium*-conditioned medium divided more rapidly than did sister cells isolated into medium which had no such conditioning. These experiments are summarised in Table II. The difference of 5.46 between the two populations is statistically significant.

Table II. *The effect on rate of increase of eight Paramecium produced by adding a small amount of homotypically conditioned medium (data from Dimitrowa).*

	Number of cultures	Mean number individuals	Mean error
Experiment	48	17.52	0.76
Control	48	12.06	0.57

Dimitrowa interprets these results as indicating that the introduction of a small amount of mild contamination of the excretion products from the species tested exerts a stimulating effect on the initial division rate. The concentration of homotypic excretory products which exhibit this stimulating effect is apparently limited to a very narrow range, while the retarding influence of such contamination is exerted over a much wider range of concentrations. This suggests one possible explanation for the contradictory conclusions obtained by some other workers, but is not sufficient to explain the divergence of the results obtained by Dimitrowa and Di Tomo. To obtain a clue to this divergence we must consider the rôle played by the bacterial flora in such cultures; this consideration can best be undertaken by reviewing the work of Johnson (1933).

Johnson found no difference in the division rate for the first 24 hours of culturing *Oxytricha fallax* singly or in pairs in one or two drops of bacterised hay infusion. Pure line cultures of 32 sister cells descended from the same individual

of a frequently re-isolated laboratory clone were used in all such experiments; half were isolated singly and half were in pairs. Cultures in five drops of bacterised hay medium showed a higher rate of reproduction when started with two *Oxytricha* than when started with but a single organism. Fourteen such series, each containing 32 animals, 16 paired and the same number isolated, at the end of 24 hours gave a mean difference of 3.7 more animals in the cultures started with two organisms. For ten-drop cultures the progeny of pairs exceeded those of the same initial number of isolated animals by an average of 1.03. Both results are statistically significant.

When closely related single *Oxytricha* from the same clone were isolated into 2, 5, 10, 20 and 40 drops of hay infusion, the mean population from 14 series at the end of 24 hours was 6.3, 5.6, 4.8, 4.1 and 3.5 respectively. The differences between any two populations are statistically significant. Changes in temperature, changes in hydrogen-ion concentration (cf. Darby, 1929, 1930) and the use of paraffined dishes, if the conditions were comparable throughout a given test, did not alter these relationships.

Johnson obtained evidence that the bacterial flora is important in determining the effects of numbers present or of volume per animal upon the rate of fission. Further experimentation showed that *Pseudomonas fluorescens* in a non-nutritive, balanced physiological salt solution formed an adequate culture medium for *Oxytricha*, other conditions being favourable. Adequate tests indicated that under these conditions the bacteria tended to decrease rather than to increase in numbers in 24 hours. With single *Oxytricha* isolated into 4X, 2X, X, X/4 and X/10 concentrations of this bacterium (X equals approximately 10^8 per c.c.) the mean results at the end of 24 hours were 3.54, 9.0, 11.32, 5.37 and 2.98 respectively. Incidentally it is worth noting that the rate of increase of *Oxytricha* was greater at X concentration of this bacterium in this medium than was shown by similar animals isolated simultaneously into the usual bacterised hay infusion. There was no difference in the reproductive rate of one and two *Oxytricha* in two drops of this optimal density during 24 hours. In two drops of supra-optimal densities, two *Oxytricha* reproduced faster than one, except in very dense suspensions where reproduction was inhibited. In sub-optimal densities, the reproductive rate of single *Oxytricha* was highest in the largest volumes of medium used. Some of these relationships are shown in Table III, which is a summary of the results from 14 series of experiments.

Table III. *Reproduction rate for 24 hours for one and two Oxytricha in two drops of different concentrations of bacteria.*

Concentration	4X		2X		X	
Number animals seeded	1	2	1	2	1	2
Mean population	4.2	5.19	8.02	10.17	10.62	10.35

The differences with 4X and 2X concentrations are statistically significant; that with X concentration is not. With 5X concentration little reproduction occurred even in cultures seeded with four animals, and several of the protozoans died. This

concentration of bacteria seems to be about the limit of density which these animals can stand. The results show that with supra-optimal populations of bacteria which are not too dense, an initial seeding of two animals has, during the early history of the culture, an advantage over a culture started with a single individual. The stimulating effect of an increase in volume, which is the converse of the experiment just reported, can also be demonstrated. Results from a series of such experiments are exhibited in Table IV.

Table IV. *The reproductive rate for 24 hours for isolated Oxytricha in 2 or 10 drops of X or X/10 concentrations of bacteria (P. fluorescens).*

Concentration	X		X/10	
Number drops	2	10	2	10
Mean population	10.17	9.87	2.92	5.62

Johnson's experience with suspensions of bacteria suggests that the results which he and others have obtained with the hay medium may be due to the concentration of bacteria present rather than to other factors. This reminds one of Robertson's remark in 1927, when, after retesting his findings in the light of criticisms from other laboratories, he states that all the data and conclusions concerning allelocatalysis in infusoria which have been issued from his laboratory remain valid, save that they may apply to the associated food organism and not to the infusoria themselves. Johnson's work does not confirm Robertson's theory of the cause of allelocatalysis, but it does confirm the existence of the phenomenon Robertson discovered and the importance of the food organisms associated with the infusoria upon their rate of reproduction.

In making comparisons with the studies dealing with hay infusions, one must assume that in the latter there are sufficient bacteria to permit reproduction among the protozoans. In small volumes of the hay medium (1-2 drops) the amount of nutritive material is much less than in the larger volumes; hence the larger volumes are able to support greater numbers of bacteria, and if the bacteria are not too numerous, more protozoans as well. In Johnson's early experiments, one drop of hay medium seeded with bacteria as described supported the progeny of one or of two *Oxytricha* for 24 hours without any significant difference in the rate of fission; at the end of 42 hours the cultures started with one animal showed the higher rate of reproduction. This seems to be due to the more rapid exhaustion of the food supply in the cultures seeded with two animals. In other similar experiments when 5- and 10-drop cultures were used, the two-animal seedings showed a higher rate of fission at the end of 24 hours; these volumes of hay medium will support greater numbers of bacteria than will a single drop. From the data obtained when using bacterial suspensions where the numbers of bacteria present were known approximately, it seems plausible to suggest that the retarding effect of bacterial crowding was occurring in Johnson's tests which showed a retarding influence in reproduction rate with the larger volumes of hay medium. The obser-

vation that dense clouds of bacteria existed at the end of 24 hours in these cultures seems to support this conclusion.

The results given in Table III are similar to those of Robertson, Yocom, Petersen and Dimitrowa, in the phases of the work of the last two which supported Robertson's phenomenon. Those given in Table V are similar to those reported by Woodruff, Myers, Calkins, Cutler and Crump, Di Tomo and by Petersen and Dimitrowa in that phase of the work of the last two which fails to support the Robertson phenomenon. The differences in results in the two tables summarising Johnson's work are due to differences in density of the bacteria in the medium. These findings suggest that the differences in results obtained by the various investigators may be due to differences in the numbers of bacteria in the cultures used.

Johnson's work leaves unsolved the question of the relative importance of bacterial as opposed to some other sort of biological conditioning through the production of excretory matter or of some *X*-substance. It does indicate that the introduction of a second infusorian may reduce the supra-optimal numbers of bacteria toward the optimum; that the introduced protozoan may introduce more suitable bacteria, or less suitable ones; in short, his work has made it necessary that in future experimental attacks on this problem the bacterial flora shall be under control.

Johnson's results appear to have been confirmed even before final publication by recent work of McPherson, Smith and Banta (1932). In the brief abstract which has appeared they record that they have reared *Paramecium caudatum* in drop cultures of oat-straw medium and find that groups of animals have a higher initial rate of fission in "strong" medium than do isolated animals; in "weak" medium the reverse is true. These workers are primarily concerned with cladoceran culture, and further report with *Moina macrocopa* reared 1, 3, 6 and 9 per 50 c.c. of culture medium, that in "concentrated culture medium" the time of first release of parthenogenetic young bears an inverse relation to the numbers in the initial seeding; in dilute medium the order is reversed and the isolated *Moina* release young first. The medium used included a manure solution and a bacterised medium. Further details are not yet available.

Previously Stuart reported that the same species of *Moina* will produce more young in a homotypically conditioned medium than in one which is unconditioned. Briefly he found that in sister lines of the same stock the mothers in the conditioned medium produced more young and more second eggs than did those in the unconditioned medium. Also the mortality in the conditioned medium was much less. These results were obtained when the conditioned or unconditioned water was used as the diluent only for the usual stock manure infusion. The factors involved in the conditioning are not yet analysed.

As regards population increase, even with available food held constant, demonstrations of the harmful effects of undercrowding are now as available as the better known harmful effects of overcrowding. Some years ago it was discovered (Allee, 1931) that in Chapman's population studies on the flour beetle, *Tribolium confusum*,

maximum rate of initial population increase occurred at a medium rather than at a minimum population. This discovery has been independently confirmed by T. Park (1932) and by MacLagan (1932). T. Park found, for example, that two pairs of adult virgin beetles seeded into 32 gm. of flour produce more eggs per female per day in the first 11 days of a culture than do a single pair, or more than two pairs so seeded.

An analysis of the dynamic factors concerned in this situation is now available from Park's work. In brief, he finds that the results are due to the interaction of two opposing factors. The first of these, cannibalism or egg eating by adult beetles, is most extreme in concentrated populations, and lowers the egg numbers therein. This relationship favours maximum population growth in the smaller populations. The second factor is revealed by the experimentally proven fact that copulation and successive recopulations stimulate the female *Tribolium* beetles to produce more eggs with a higher percentage of fertility; this factor favours the more rapid population increase in the more dense populations, since it has again been proven experimentally that contacts between the sexes in these beetles is a direct function of the population density. The cannibalistic effect stands in direct proportion to the numbers present; the copulation and recopulation effect is proportionately greater at low than at high population densities. The interaction of these two opposing factors results in an intermediate optimal population in which females produce significantly more fertile eggs than do those from either smaller or larger populations.

The grain weevil, *Calandra granaria*. (MacLagan, 1932 *a*), reproduces most rapidly when 400 grains of wheat are present per female weevil. There are indications that with an increase of the grains present per female to 800 or even to 600, a reduction in the number of progeny follows. This means that with *Calandra*, as with *Tribolium*, there is an optimum density for reproduction. No analysis of the factors involved in this reduction in reproduction rate with undercrowding of *Calandra* has been presented.

VIII. EFFECTS OF NUMBERS PRESENT ON OXYGEN CONSUMPTION.

Earlier studies (Allee, 1931) have shown that the rate of oxygen consumption forms a fairly delicate test for mass effects. For aquatic animals, Winkler's method of determining oxygen has been much used in this connection. Recent tests (Allee and Fowler, 1932) have shown that as used in our laboratory, repeated samples from the same water yield, under careful manipulation, means with a standard error of ± 0.009 c.c. per litre; with the less rigorous procedure too likely to be characteristic in a long series of routine determinations, the error increased to ± 0.036 per litre. These tests show the degree of repeatability we have obtained in ten successive trials. Variations in manipulation aside, an error to be avoided in the use of the Winkler method is the possibility that biologically conditioned water may adsorb iodine and accordingly introduce a fundamental error. Goldfishes are known to give off a considerable amount of mucus into the water in which they

live; a litre of water in which four medium-sized goldfishes had lived for 30 hours showed an iodine error equivalent to 0.023 c.c. of oxygen per litre. The experiments to be reviewed showed differences consistently over the combined limit of error just indicated before they were considered to yield positive results.

The earlier studies just mentioned showed that the aquatic isopod *Asellus communis*, four different species of land isopods, the cladoceran *Daphnia pulex*, and the ophiurid *Ophioderma brevispina*, all showed a lowered rate of respiratory exchange following the formation of an aggregation, as compared with the rate of oxygen consumption or carbon dioxide production of isolated animals. This initial decrease in the rate of oxygen consumption is not due to a lowered oxygen tension of the medium surrounding the grouped, as compared with the isolated animals. *Ophioderma*, out of the breeding season, allowed to starve in water changed each 24 or 48 hours, show first a period in which the grouped animals are using less oxygen per individual than those isolated, after which the grouped animals use oxygen at a higher rate than do the accompanying isolated controls.

During the summer, however, the initial formation of the aggregations results in an increase in the rate of oxygen used, and in the breeding season there is no steady marked difference in the oxygen consumption of grouped and isolated animals except for occasional periods of unusually high rates, which were found more frequently among the grouped than among the isolated individuals (Allee and Fowler, 1932).

As a result of earlier studies upon oxygen consumption of grouped and isolated *Ophioderma*, the suggestion was made that the physiological effect of aggregation in these animals is, at least in large part, due to the physical substitution of other members of the group for the missing elements in the environment. Obviously it is somewhat difficult to secure evidence upon this point during the breeding season, but by comparing the oxygen consumption and the autotomy of grouped and isolated animals in plain containers and in containers holding also loose heaps of glass rods, decided similarities were discovered between the effects produced by close aggregations of groups of starfishes and by the contact between an isolated starfish and the glass rods introduced to simulate somewhat the eel grass upon which the animals normally live. This conclusion is supported by the observation that in the presence of glass rods there is a reduced tendency of the ophiurids tested to collect in close aggregations; this supports previous observations that as laboratory conditions approach those in nature, the aggregations formed in the laboratory become less common and less compact.

Schuett (1933) states that with the fishes, *Lebistes reticulatus*, *Umbra limi*, *Carassius auratus*, and *Fundulus heteroclitus*, when four fishes are present in a given volume of water the amount of oxygen consumed per fish is lower than is the amount used by a fish isolated into the same volume of water. The same group effect occurs in goldfishes in flowing and in non-flowing water. When the volume per fish is the same for the grouped and isolated fishes, the difference in oxygen used in quiet water, the only condition tested, becomes insignificant. This effect has been confirmed for goldfishes by Bowen (1932). These workers did not correct

for a possible nitrite error which is now believed to have affected both Schuett's and Bowen's work with goldfishes and may account for the differences in oxygen consumption observed with these animals when grouped and isolated.

Working with the closely schooling *Ameiurus melas*, Bowen found the opposite effect; the grouped bullheads used more oxygen per individual than did the accompanying isolated controls. The difference in the respiratory behaviour of goldfishes and bullheads is correlated with the different behaviour of the two types of fishes when aggregated. With the goldfishes, Bowen and Schuett found no observed difference in the swimming behaviour of the grouped and isolated fishes. Welty, from a longer contemplation of the behaviour of these fishes, reports that the group fishes are less likely to sudden starts and are more apt to engage in steady exploratory movements. The *Ameiurus*, on the other hand, clearly act differently when grouped than when isolated; if aggregated each individual tends to push in toward the centre of the group and the obviously increased activity is reflected immediately in the increased oxygen consumption on the part of members of the group. The possible nitrite error would increase the observed difference.

These respiration studies have yielded up to date two types of effects of groups of animals upon the rate of respiration, and the two are diametrically opposed; in one type, now represented by a number of animals of widely different taxonomic position, the group depresses the rate of oxygen consumption; in the other, clearly represented by *Ameiurus*, the group stimulates oxygen consumption. In the brittle starfish, *Ophioderma*, initial group stimulation during the breeding season gives way to initial group depression after the breeding season closes. Some of the survival significance that may be correlated with the group effect on oxygen consumption is indicated by the work of Fowler (1931), who showed that the cladoceran, *Daphnia pulex*, lives longer in various relatively concentrated solutions of electrolytes when present in numbers than when isolated. In a further analysis of the underlying causes of this increased longevity on the part of the group, Fowler found that with calcium chloride, the grouped individuals showed a decreased rate of oxygen consumption as compared with the isolated animals. The grouped animals were shown to give off as a group significantly larger amounts of carbon dioxide than did any one individual; this increased carbon dioxide tension, probably by its effect on activity, caused a decrease in the rate of oxygen consumption of *Daphnia* and an accompanying decrease in the sensitivity to the toxic effects of relatively concentrated solutions of the salt.

IX. MASS PROTECTION FROM TOXIC CONDITIONS.

There is much evidence, a sample of which has just been given, to show that frequently a group of animals will yield each other protection from a toxic environment to which animals exposed singly would succumb. Two sorts of toxicity have been investigated with relation to such mass protection: that caused by the absence of needed materials, as when marine animals are exposed to fresh water, and that

caused by the addition of some toxic substance, such as colloidal silver. Mass protection from these two types of toxic conditions will be reviewed in the order given.

The marine turbellarian flatworm, *Procerodes wheatlandi*, lives in the intertidal and adtidal¹ zones in restricted regions along the coast of New England. They will survive longer in fresh water, other conditions being equal, (a) if they are present in numbers, (b) if isolated into fresh water in which other living *Procerodes* have previously been exposed, and especially (c) if isolated into fresh water in which some few *Procerodes* have recently died and disintegrated, even though such a conditioned medium be boiled and filtered, and (d) if the salinity of the fresh water is increased by adding as much as 0.5 per cent. of sea water. The protective material will stand boiling and will pass through filter paper (Allee, 1931).

The protective value of this biologically conditioned fresh water is maintained after dialysis to the same electrical resistance as that of extremely dilute sea-water controls. Water extracts of marine amphipods and of the fresh-water planarian, *Planaria maculata*, show the same effects as water from cultures of the latter and heated hay infusions from a practically pure culture of *Paramecium*.

Further studies (Allee, 1933) concerning the action of hypotonic water extracts of *Procerodes* and of *Planaria maculata* culture water on the marine *Procerodes* isolated therein have confirmed the essential results of these earlier investigations and in addition have furnished evidence as follows:

Adsorption on charcoal or on egg albumen does not significantly affect the protection furnished by planarian culture medium. No protection is furnished by egg albumen, gum arabic, gelatine, or mucus made by dissolving dried parotid gland in water. Depressants, such as ethyl alcohol or ethyl urethane, have no protective value. Water solutions made from ether-soluble and ether-insoluble fractions of *Procerodes* extracts exhibit less protection than does the whole water extract. The protective effects are not due to difference in pH within the limits tested.

Cane-sugar solutions definitely confer protection in all concentrations tried, from $M/1$ to $M/33\frac{1}{3}$. Solutions of $M/40$ and $M/50$ of cane sugar gave the same survival as did the non-sugar controls. The protection conferred in these experiments by $M/25$ and $M/33\frac{1}{3}$ solutions was approximately of the same order as that given in accompanying isolations into planarian culture water. This culture water did not possess osmotic pressure beyond that furnished by electrolytes, and these were balanced against the electrolytes in the controls in so far as this can be done, by testing their conductivity.

While these experiments reinforce and extend the essential conclusions of the earlier work they do not throw light on the nature of the protective mechanisms involved.

For some years there has been no further need of experimentation concerning the possibility of securing mass protection for animals exposed to the toxic action of colloidal silver; but there has been need of a careful analysis of the mechanism

¹ Region adjacent to and just below low tide.

or mechanisms involved in the readily demonstrated group protection. The pertinent possibilities suggested up to date include the following, which are by no means mutually exclusive:

(1) The group more effectively produces an autoprotective substance which in some manner conditions the medium and protects the group (Drzewina and Bohn, 1921, 1928).

(2) The larger number of animals more rapidly adsorbs or exhausts the toxic agent, or, on the other hand, each animal receives a smaller dose (Bresslau, 1924; Allee and Schuett, 1927; Carpenter, 1927; Breukelman, 1932).

(3) The grouped animals tend to show a reduction in the rate of metabolism as measured by the oxygen consumption compared with isolated animals during the early stages of their association, and this reduction favours survival in toxic solutions at certain concentrations.

(4) The group may be protected by altering the electrical conditions (Drzewina and Bohn, 1926), or, as these workers state in 1928, animals may be mutually influenced by the presence of other animals without the diffusion of any substances, by "catalysis by contact."

If we limit the discussion as to which of these suggested mechanisms has been working in the protection of grouped goldfishes from the toxic action of colloidal silver (Allee and Bowen, 1932) it is found that (1) it is possible to account quantitatively for the colloidal silver introduced into the different lots of animals, and that the suspensions containing grouped animals have had significantly more silver removed than have the suspensions which contained isolated fishes. (2) With volume and silver dosage identical per individual fish, there is no significant difference in survival; that is, there is no significant evidence of group protection under such conditions. (3) In processionary series (Carpenter, 1927) the fishes introduced later in the series survive longer than those introduced earlier. If no chemical analyses are made, such results can be interpreted as supporting the hypothesis of protection as a result of the gradual accumulation of an autoprotective substance. Since, however, we know that with related experiments the conditioned water removes the toxic colloidal silver from suspension, it is very probable that the same thing happens in a processionary series. (4) Colloidal silver suspensions in which a number of fishes have lived for 24 hours showed in chemical analyses that by the time the analyses were made all the free colloidal silver had been removed from a 3 per cent. conditioned suspension, and that if more colloidal silver were added to such a conditioned suspension, a part of it also would be adsorbed. On the other hand almost no free silver was lost from unconditioned suspensions containing a single fish.

All of the evidence collected in this laboratory concerning group resistance to colloidal silver favours the assumption that the major portion of the protection is due to the more rapid removal of the silver by the massed individuals or their products. The question remains whether all the observed protection can be attributed to the working of this factor; the evidence at hand indicates that no other factor need be called on; but there are so many cases where the same end results

are obtained by more than one method that there may be and probably are other protective devices in these cases. The protection furnished by reduced metabolism should be as effective in safeguarding grouped goldfishes from colloidal silver as in protecting *Daphnia* from sodium chloride (Fowler, 1931).

Such possible group protection with goldfishes is not due to the presence of an increased amount of carbon dioxide, for in our experiments with colloidal silver, the protection in acid and in alkaline medium was the same, though the effect of carbon dioxide should differ in the two. Further, the mass protection was found in colloidal silver suspensions with the initial pH at 4.3, 7.0 and 9.6.

Of the two mechanisms which may be acting, the greater removal of toxic colloidal silver by the grouped fishes and the lower rate of general metabolism in the group, it would appear that the former is much the more important. Of the substances given off by the fishes, slime is clearly effective in fixing colloidal silver. A synthetic fish urine also throws colloidal silver out of suspension but not so efficiently as does adsorption on slime. Faeces are probably effective both by direct adsorption of silver and by contributing salts to the medium. No one of these is specifically autoprotective in the sense used by Drzewina and Bohn. There is no critical evidence available regarding the possibility of electrical changes in the medium produced by numbers of animals. Magrou, Magrou and Reiss (1931) in their discussion of this possibility impose the important condition that there must be electrical isolation between activator and recipient. The suggestion that electrical changes occur associated with numbers present has also been made independently and vaguely by Podhradsky (1932) in his work on mass effects on growth in tadpoles. *A priori* the suggestion of the importance of biophysical relations in mass physiology has merit, but the extent of its application, if at all, is not yet apparent.

X. MORPHOLOGICAL EFFECTS.

Polarity is one of the fundamental properties of almost all organisms. In some plants, including the algae *Fucus* and *Ascophyllum*, polarity can be readily induced in the fertilised egg by exposure to an asymmetrical environment, particularly by differential exposure to light, as was originally shown for *Equisetum* eggs by Stahl (1885). In testing for this effect on eggs of *Fucus* and *Ascophyllum*, Rosenvinge (1889) found that if the eggs are grouped the first cleavage tends to develop at right angles to an adjacent egg or to the line from the centre of a group of eggs. The first cleavage separates the egg of these plants into unequal cells, one of which is the apical cell and the other is the rhizoidal; this is the first outward evidence of the future axis of the plant.

Hurd (1920) reinvestigated this subject with the eggs of *Fucus inflatus* (?). While the group effect can be noted even when the eggs are under the influence of unilateral illumination, it is most readily shown when they are in darkness. When the *Fucus* eggs are within 0.2–0.3 mm. of each other, groups of 2–4 eggs will show the phenomenon as well as will masses of 50–100. Frequently radiating designs are formed, striking in appearance; the rhizoids grow in toward the centre of the group regardless of the rearrangement of the eggs before germination.

Whitaker (1931) has reinvestigated this group effect. Using *Fucus vesiculosus* eggs, he found that when only two of these were placed 1–3 egg diameters apart in darkness and under favourable experimental conditions, polarity of the eggs was not affected. When large compact masses of eggs were placed in the centre of a dish and others were placed around the periphery of the mass and about two egg diameters away from it, clear-cut positive results were obtained. For example, in one experiment 257 eggs showed rhizoids directed towards the central mass and only two showed them oriented in the opposite direction. The eggs in the mass also developed with a similar orientation save that the eggs in the centre tend to divide to form equal and similar cells. In larger dishes, supposedly with more water present, more eggs are necessary to produce this effect than in smaller dishes.

Eggs of *Ascophyllum* and of the two species of *Fucus* tested, all show this group effect though not necessarily to the same degree. Eggs of *F. vesiculosus* are less sensitive to group influence in this regard than are those of *F. evanescens*. The effect is not species specific, for unfertilised eggs of the former species of *Fucus* are able to control the developing polarity of eggs of the latter when the *F. evanescens* eggs are placed, well separated from each other, around or on one side of a mass of *F. vesiculosus* eggs. In seven such experiments, all carried on under controlled conditions and in darkness, 133 of the *F. evanescens* eggs developed rhizoids directed toward the heterotypic unfertilised eggs, two showed reversed orientation, three developed tangentially and four showed equal divisions.

Since these results were obtained as a result of proximity to a mass of unfertilised resting eggs of a different species, they cannot be species specific; neither are they due to the action of some force or forces released during cell division. Rosenvinge, years ago, suggested that the effect is due to the presence of an environmental gradient of oxygen or of nutritive materials. Winkler (1900) showed that with *Cystosira barbata* an artificially produced oxygen gradient did not affect the polarity of the developing egg. Despite Winkler's apparently sound work for the species used, and because of the known variation in the effect of light in determining the polarity of eggs of related species of plants, the whole matter of the interpretation of this well-observed group effect awaits investigation; in such an investigation the possibility of biophysical as well as of biochemical gradients must not be overlooked.

XI. CROWDING AND THE PHASE THEORY OF LOCUSTS.

With animals, physiological effects register readily as changes in function of the whole or of parts of the individual; they are much less readily shown by definite changes in structure. One of the outstanding instances of the suggested expression of mass physiology in morphological terms is to be found in Uvarov's theory of phases in migratory locusts, which is ably summarised in his 1928 book. The phase theory states that species formerly supposed to be monomorphic are in fact polymorphic, as regards coloration of the nymphs and the form of the pronotum, body size and relative length of wings of the adults.

After considering the evidence for this theory at some length in 1931, I con-

cluded that while the evidence presented did suggest the transformation of locusts from one phase to another, according to the degree of crowding, such a transformation was not actually demonstrated. The evidence at hand at that time was strong enough to have become the basis of operation of locust-control officers, but it was not critically convincing and the phase theory was left with a verdict of unproven, but a promising opening for further work.

Vayssi re almost immediately (1931) presented some slight additional evidence that transformation from one phase to another can be induced in the laboratory. Faure (1932) has been able to present in detailed form his extensive and convincing observations which supply the needed critical evidence supporting the phase theory of locusts. In the course of this work, Faure took hoppers (nymphs) of the phase *solitaria* of *Locustana pardalina* in the field and subjected their progeny to crowding in his experimental cages at Pretoria. Adults of the same phase were also collected in the field and their progeny were crowded in the cages, as were also young hoppers of this phase, collected in the veldt.

The results of this crowding were judged by its effect upon the behaviour and coloration of the nymphs and upon the biometrical measurements of the adults. These results showed that crowding the solitary nymphs, if begun early enough, whether they had been taken directly from the open country or from controlled laboratory cultures, would produce changes in activity and coloration such that many of the nymphs, frequently a majority of all treated, came to resemble typical specimens of the phase *gregaria*. Others developed an intermediate condition.

The biometrical data reveal that in most cases the cage-bred adults which were measured showed absolute and relative characteristics which were nearer to the phase *gregaria* than to *solitaria*. Suggestive as these results are, I find the experiments made to test the hypothesis that Mendelian inheritance is the cause of differences in coloration of the grasshopper nymphs to be more convincing evidence favouring Uvarov's theory of locust phases. In these experiments the parents were selected in the fifth instar as typical examples of phases *solitaria* or *gregaria* of *L. pardalina*. Egg masses were obtained and hatched and the progeny of the same mating were reared partly in isolation and partly in small crowds, with the results in each case noted separately. Isolation here means complete isolation with one nymph to the container. The data are summarised in Table V.

Table V. *The results of rearing grasshopper nymphs of identical parentage, partly in isolation and partly in crowds.*

Mating	Number pairs	Number nymphs hatched	Results					
			Isolated			Crowded		
			<i>Sol.</i>	<i>Trans.</i>	<i>Greg.</i>	<i>Sol.</i>	<i>Trans.</i>	<i>Greg.</i>
<i>Gregaria</i> × <i>gregaria</i>	6	412	111	1	1	5	16	121
<i>Solitaria</i> × <i>solitaria</i>	7	113	51	0	0	0	4	14
<i>Solitaria</i> × <i>solitaria</i>	10	431	113	0	0	11	5	54
<i>Gregaria</i> × <i>gregaria</i>	9	341	90	0	0	11	1	1
Totals	32	1297	365	1	1	27	26	190

Among the nymphs reared in isolation, the one which was classified as phase *gregaria* when in the fifth instar had been in a cage with ten others during the first instar and was isolated when in the second instar.

All the parents used in these mass matings came from the laboratory cages with the exception of half of those used in the third series; in five of the ten matings of this series, both the males and females came from phase *solitaria* nymphs collected in the field in the fifth instar. The parents used in the second series were from the F_1 generation of the first series reported. They had been reared in isolation and were phenotypically phase *solitaria* from phenotypically phase *gregaria* parents.

According to Potgieter (1929) the phase *gregaria* is a Mendelian dominant and phase *solitaria* is a Mendelian recessive. The breeding experiments do not bear out this conclusion and do indicate that the coloration of the nymphs is determined by crowding or the lack of it.

Observations were carried further and comparisons of adults from these matings were made on the basis of the relationships existing between the crowding and isolation of nymphs and the structural proportions of imagos. These data from a limited series of progeny selected at random from two matings showed that, of the four comparisons made, in all cases the males reared in isolation approached the *solitaria* condition, while those reared under crowded conditions exhibited ratios tending in the *gregaria* direction. With females, the results of one mating were entirely similar to those with males; in the other mating, only one of the comparisons used showed results according to the expectations of the phase theory.

While these biometrical results support in general the phase theory, the paucity of the data and the irregularity of the results with females do not carry the conviction that is given by the results based upon changes in the coloration of the nymphs. This is unfortunate, since the biometrical data are concerned with structural relations which are less easily affected by environmental influences than is colour. Even so, taken with the biometrical data based on mass crowding experiments, there is little room for doubt that crowding does effect changes from the morphological aspects characteristic of one phase toward an approximation of the other.

In order to account for these effects of crowding, Faure advances two hypotheses. The first accounts for the coloration of the nymphs, the second for the structural changes of the adults. The black coloration characteristic of nymphs of phase *gregaria* is largely due to cuticular pigmentation. Some of the orange, also a characteristic colour of the phase *gregaria*, is also to be found in the cuticula. Crowding results in a great increase in activity and must affect the rate of metabolism of the entire organism. Under these conditions the muscles and other tissues produce more excretory products than can be dispersed by the Malpighian tubules, and they or their derivatives are deposited in the cuticula and are shed in moulting.

Faure proposes the name *locustine* for these unknown products of a high rate of metabolism, and briefly summarises his hypothesis as follows: "The amount of orange and black" (in the coloration of nymphs) "is in direct proportion to the amount of locustine produced by the organism." Faure suggests that the assump-

tion of the typical phase *gregaria* coloration within an hour after hatching is due to the transmission of locustine from the mother through the egg-yolk. If the nymph is crowded and hence active, it produces its own locustine and continues in the orange and black coloration from instar to instar. If solitary and inactive, locustine is not produced in sufficient amounts and the nymph on moulting tends to assume the colour characteristics of phase *solitaria*.

This theory can be tested by comparing the coloration of newly hatched nymphs from the mating of *gregaria* females with *solitaria* males, with that of similar nymphs from the reciprocal cross. Presumably, if the hypothesis holds, the former would on hatching be orange and black and the latter would lack these colours. Data are not presented in Faure's extensive report which would allow the immediate application of this test.

The morphological characters of phase *gregaria* are also an expression of its activity, but here Faure doubts the importance of locustine and suggests the possibility of the strains and stresses accompanying the extreme muscular activity characteristic of nymphs of this phase producing the alteration in form of the pronotum. He thinks that the greater length of the wings in phase *gregaria* is due to the greater muscular activity in the thorax during the early stages of their development.

Faure concludes this discussion (p. 368): "In a sense the *gregaria* characteristics might be described as the external evidence of semi-pathological conditions resulting from excessive metabolism; the orange and black are due to metabolic products deposited in the cuticula and the pinched pronotum and long wings are modifications brought about primarily by excessive muscular exercise."

XII. THE CONTROL OF SEX IN CLADOCERA.

The control of the change from parthenogenetic to sexual reproduction in Cladocera has been reviewed previously (Shull, 1929; Allee, 1931), but in order to obtain a clear view of the recent development of the problem it appears advisable to sketch briefly the general background. Stimulated by the contention of Weismann (1879) that the succession of bisexual and parthenogenetic forms in Cladocera is due to an internal rhythm, Grosvenor began experiments in 1904 in the course of which (Grosvenor and Smith, 1913) it was discovered that if the parthenogenetic females of *Moina rectirostris* were kept isolated in separate vessels from birth, they produced a much smaller proportion of males than did sister individuals which were crowded. While there was also an effect of temperature upon male production, crowding increased significantly the percentage of males at all temperatures tested.

With regard to the mechanism whereby the effect was produced, Grosvenor and Smith at first attributed the phenomenon to the accumulation of excretory products in the culture medium. Later experiments, however, showed that these excretory products either are very unstable or they are not to be considered as the agents initiating the change in type of reproduction. Accordingly they sug-

gested the possibility that the results were due to a difference in the amount of available food, but were unable to decide the question from the experiments performed.

At the time of the publication of the Grosvenor and Smith report there was already a considerable literature bearing on the possibility of parthenogenetic reproduction being changed to bisexual by means of external influences. In fact, Lubbock had suggested in 1857 that sex in Cladocera is environmentally determined. It is not the function of this review to consider all the factors, but rather to trace briefly the discussion in its bearing on mass physiology. As early as 1892 de Kerhervé reported experiments indicating that nutrition is important in maintaining parthenogenesis and that poor nutrition tends to be followed by the production of bisexual forms. Issakowitsch (1905), experimenting on *Simocephalus*, concluded that the appearance of bisexual forms is affected by food and by temperature, the latter working through the former. Woltereck (1909), using *Hyalodaphnia*, was able, within limits, to control the appearance of bisexual forms by changing the amount of food, and (1911) expresses doubt as to the importance of changes in the chemical nature of the medium except through an indirect effect upon the food supply. Papanicolaou (1910), with *Simocephalus*, found that at favourable temperature and at the labile period in the reproductive cycle, repeated transfers to fresh culture water rich in food postponed the appearance of bisexual forms. Later (1910 a), also with *Simocephalus*, he found no effect of metabolic waste products upon the change from parthenogenetic to bisexual reproduction, but thought that these served only to decrease the numbers in the population. Papanicolaou concludes from reviewing the evidence to date that food (and temperature) are not without effect on the reproductive cycle.

McClendon (1910), using *Daphnia pulex*, found that excretory products, food and temperature affected the appearance of bisexual forms. He concludes that nutrition is the most important environmental factor concerned.

Tauson (1930) also concluded that food plays an important rôle in the presence or absence of bisexual reproduction of *Daphnia pulex*. Its significance is not qualitative but quantitative. The lack of food calls forth the appearance of males and ephippial females; rich food induces parthenogenesis. Tauson also analyses and emphasises the importance of temperature and especially of pH in determining the type of reproduction in *Daphnia*.

On the other hand Kurz (1874) obtained males in a number of species of Cladocera as a result of a high degree of evaporation of the medium and as a result of fouling and of unfavourable temperature. He reported that different species are affected in different degrees by these environmental influences. Langhans (1909), after culturing four different species of *Daphnia*, suggested that the appearance of males and sexual females is due to the accumulation of metabolic waste products in the water. He advanced no evidence bearing directly on this point, but based his suggestion on other effects he had observed in cultures supplied with excess food, and which he had interpreted as being due to the accumulation of metabolic wastes.

Smith (1915) reported that *D. pulex* showed an increased percentage of males as a result of crowding, though not to the same extent that he and Grosvenor had found earlier with *Moina*. Unlike the latter, which feeds on bacteria, *D. pulex* feeds on algae. Smith grew them in cultures of *Protococcus* in the presence of an excess of food. He concludes that with this species "there can be no doubt that the way this factor (crowding) exerts its effect is through the presence of some excretory materials in minute quantities...."

Under conditions most favourable for growth and a continuation of parthenogenesis, namely high temperature and isolation, Smith reported that during parthenogenesis *D. pulex* stores reserve material as glycogen. In crowded cultures, at 10–17° C., these animals store fat in place of glycogen. The mechanism producing this change in food storage is unknown. Smith thought it causally related to the production of sexual forms, and believed that in his cultures the change was not due to food shortage. He attributed it rather to the accumulation of some excretory product in the water. Berg (1931) found that in *D. cucullata* the production of fat globules is greatest during periods of vigorous parthenogenesis.

Hartmann (1921), interested primarily in cytological relationships, records that both an accumulation of excretory products and a lack of food (and certain other external factors) tend to bring on a depression in cladoceran cultures which often results in the appearance of sexual forms.

Not all the reports on this problem during the period we have been covering were agreed that the transfer from parthenogenetic to bisexual reproduction is brought about by changes in external conditions. Since we are dealing here with a review of the progress in mass physiology, and since the evidence still to be mentioned demonstrates that at least for certain species of cladocerans this transfer is environmentally controlled, there is at present no need for reviewing the negative evidence.

It was at this point that Banta and Brown began their work which is still in progress. The published results of their investigations, in so far as they bear on our problem, have been reviewed in some detail elsewhere (Allee, 1931). More recently they have investigated some of the interrelations between temperature and crowding in the change from parthenogenetic to bisexual reproduction in *Moina macrocopa*. Without being crowded, *Moina* females will produce male offspring at suitable temperatures. At a temperature of 14–21° C. crowding of the mothers increases the percentage of males. Immediately above and below this temperature range the maximum male production occurs with lessened crowding. At 30° C. there is a partial return to the direct effect of crowding. In view of work to be reported later one is justified in wondering as to the extent to which the temperature effects noted are direct, and the extent to which temperature acts indirectly by its effect upon the bacterial food of the cladocerans.

In general Banta and Brown found, as had Grosvenor and Smith (1913), that crowding increases greatly the percentage of males produced. This they attribute as Smith did (1915) to the accumulation of excretory products, which they find to lack species specificity, to be non-volatile and non-stable. They found also that

conditions which tend to retard the rate of development of the mothers increase the percentage of males and *vice versa*. Their most recent experiments on this point (Banta and Brown, 1930) show that when the mothers are moderately crowded, treatment with known depressing agents had the effect summarised in Table VI.

Table VI. *The effect of depressing agents on retardation of release of young as compared with time of release in untreated controls and upon the percentage of males in Moina macrocopa. Data from Banta and Brown; statistical significance for paired experiments calculated by Allee using Student's method.*

Number exps.	Control		Reagent	Experimental results		Statistical* probability of difference	Retardation in hours
	Young sexed	% males		Young sexed	% males		
106	9407	18.9	Chloretone	5784	35.0	0.015	0.935
36	2624	30.5	Phenyl urethane	3451	58.8	0.0316	0.69
26	2411	9.3	Ethyl urethane	1872	17.3	0.0804	—
92	9511	13.4	KCN	11093	18.7	0.063	1.33

* A statistical value of 0.05 is usually taken as the upper limit of significance.

Data are not available to calculate the statistical probability of the retardation effects. With regard to the increase in percentage of males the calculations given show that with two of the four drugs used the differences are above the limit usually taken as indicating statistical significance.

With the case for the effects of excretory products upon male production resting at this point pending the publication of further work, we may turn to the most recent developments regarding the effect of changes in nutrition: Banta and Brown (1929 *a*) reported experiments showing that small doses of ethyl alcohol, filtrates of adrenal cortex, thyroid, thymus and muscle tissue increase the rate of development of *Moina*. This increased rate of development counteracts the normally retarding effects of crowding and accompanies or acts to reduce the percentage of males. Since extract of dried muscle has the same effect as those of adrenal cortex and thyroid they conclude that these endocrine substances show no specific effect on the rate of development, but that the observed changes are probably due to changes in the bacterial flora which serves as food for the developing *Moina*.

Berg (1931) verified the effect of crowding on the change from parthenogenetic to bisexual reproduction. His analysis of the causal factors operating proceeds little further than the statement that bisexual reproduction is a "manifestation of a state of depression in the *Daphnia* population during the transition from parthenogenesis to gametogenesis."

In association with Banta, who is a veteran in cladoceran culture, Stuart, a seasoned bacteriologist, undertook to study the effects upon male production in *Moina macrocopa* of varying the numbers of available bacteria (Stuart and Banta, 1931), with results shown in Fig. 2.

These show a striking correspondence between bacterial count and male production, with the percentage of males increasing as the number of bacteria present decrease up to a given point, and decreasing again as bacterial numbers increase. These workers also found that the bacteria did not act by adsorption of excretory wastes upon the bacteria or upon their products. Culture media bacterised with *Aerobacter aerogenes* produced no males when the offspring of sister cladocerans

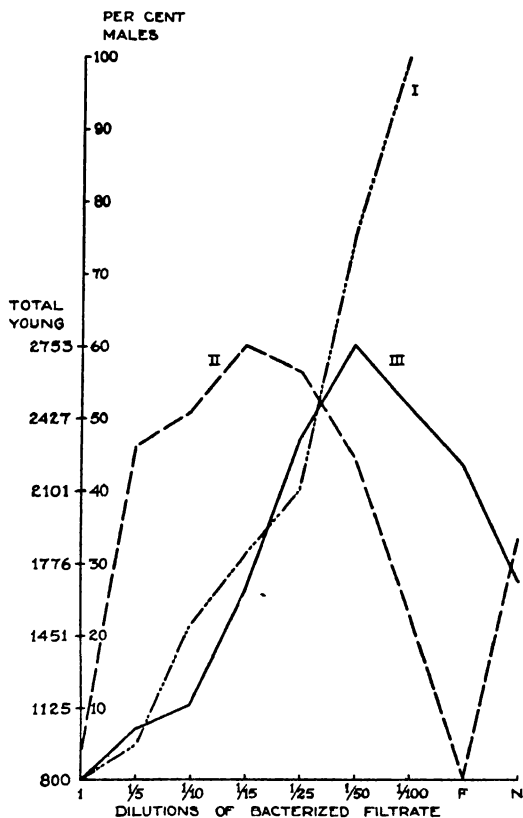


Fig. 2. Relation between dilution of bacterised filtrate and number and sex of young produced. I, male percentage of a single series; II, total number of young produced in 22 experiments; III, average percentage of males. (From Stuart and Banta, 1931.)

in normal media were producing 49 per cent. males out of 1364 young examined. They also found that *Moina* females even in crowded conditions (three cladocerans per 10 ml.) would produce only female young in the presence of an adequate number of usable bacteria.

Banta and Brown (1931) undertook to assay the relative importance of the quantity of available food and the accumulation of excretory products as factors in influencing male production in *Moina*. In the experimental series the numbers of bacteria in the bottles with isolated females ranged during the critical period for

male determination from 1.2 to 25.4 millions per c.c. In the bottles started with six females the range at the same time was from 1.0 to 14.6 millions of bacteria per c.c. No males were produced in the former and "a considerable percentage" of males were produced in the latter. The number of bacteria present do not seem to have been the male determining factor in these tests.

Later Stuart and Cooper (1932) undertook to rear *Moina* under conditions in which the intervention of excretory products would be ruled out of their experiments upon the effects of crowding on the control of the appearance of males. In their most convincing experiments they arranged an irrigating system such that the test animals were constantly bathed in a flowing medium that was constantly agitated, and hence avoided the collection of excretory products. In such irrigated experiments ten parthenogenetic females, so-called sister-mothers, were crowded in 80 ml. of liquid, and reared to egg production in a total of from 200 to 850 ml. of constantly changing medium. Of the 6685 young produced, 26.3 per cent. were males, and males appeared in 85 per cent. of the experiments. In unirrigated experiments, using the same medium and the same experimental conditions, of 4242 young sexed, 50.4 per cent. were males; these occurred in 86.6 per cent. of the experiments. The statistical probability of the difference in the percentage of males in these two experiments being significant is 0.0004. Values less than 0.01 are certainly statistically significant. With isolated mothers in their normal medium, of 5122 young examined 10.37 per cent. were males, showing that the higher percentage of males was not inherent in the condition of the brood at the time of the experiment.

The significantly higher percentage of males in the non-irrigated experiment may be due in part to the accumulation of excretory products, although the increased amount of food and the mechanical agitation of the irrigated experiments would tend to decrease male production. The experiments do show that by controlling nutrition, and with the possibility eliminated of the accumulation of excretory products of *Moina* or of the materials produced by their decomposition, a high percentage of males can still be produced by crowding prospective mothers, though isolated sisters are not producing males. This conclusion is supported by experiments in which the culture medium was changed frequently, in contrast with simultaneous experiments in which there was no such change.

More recently Stuart, Cooper and Miller (1932) point out that the demonstration that excretory products are causally related to the production of male offspring in Cladocera is difficult of proof, since any attempt to study the physico-chemical nature of the excretory products demands manipulation, which as experiments of Banta and Brown indicate, destroys their efficacy as sex-controlling agents. This makes the rôle of excretory products in sex determination in Cladocera subject to an indirect attack only.

Stuart and his associates investigated the possibility that aeration of the medium by shaking decreased the percentage of males among the offspring by some means other than the effect of the shaking on the contained excretory matter. By appropriate experiments they discovered that shaking the mothers at hourly intervals during the period when the sex of the offspring was labile, decreases significantly

the percentage of males produced. The effect was almost as great when the mothers were shaken and then transferred to undisturbed media for the intervals between shaking, as when they were shaken with the medium in which they live. Shaking the mothers resulted in an 85 per cent. decrease in male production, while shaking the medium and not the mothers resulted in a decrease of only 33 per cent. The comparisons are with the progeny of unshaken crowded controls containing sisters of the experimental animals.

Crowded mothers in normal medium would, in these experiments, be expected to give 36 per cent. males; if shaken, the cladocerans presumably had their metabolism increased, and gave but 5 per cent. males. Mothers reared in a medium poor in food yield only female young; by shaking them their metabolism should be increased and some males should result. Experiments showed a 69 per cent. increase in males resulted from this treatment. These experiments can be explained by the combined food-metabolism hypothesis and are in accord with the relation between bacterial numbers and sex.

Both Banta and Brown (1929) and Stuart, Cooper and Coady (1932) find that carbon dioxide, which is the best known product of metabolism of these animals and which Fowler (1931) has found plays an important rôle in protecting groups of *Daphnia* from the toxic effects of solutions of electrolytes, if added to the cultures of crowded cladoceran mothers, practically suppressed male production.

Despite all the evidence at hand concerning the lability of sex in Cladocera, Stuart, Cooper and Miller (1932) find, especially in the so-called depression periods, that there are a considerable number of "sex-fast" individuals not readily, or perhaps not at all amenable to environmental influences as regards bisexual production. The factors underlying both "sex-fastness" and these "depressions" are not yet analysed.

The work of Stuart and his associates does not rule out the possibility of control of male appearance in cultures of *Moina* by the accumulation of metabolic products, even though they themselves have come to doubt the efficacy of such metabolic wastes. They do demonstrate that under appropriate conditions parthenogenetic can be changed to bisexual reproduction by controlling nutrition; Banta and Brown's experiments (1931) show that sex in *Moina* is not always controlled so. Their experiments further suggest the possibility of a food accessory factor playing some rôle in this change, and that there may be some other sort of group effect, not yet analysed, which has some influence in controlling the appearance of males. The light thrown on this problem by the efforts of this group of trained bacteriologists illustrates the other advances that may be expected in mass physiology and other aspects of modern biology, if and when bacteriologists become less completely engrossed with medical and economic aspects of their science.

XIII. GROUP INFLUENCE ON BEHAVIOUR.

There is nothing novel in the idea that grouped animals behave differently from isolated ones (Tarde, 1903), but the precise effect of numbers of animals present upon the rate of conditioning a new behaviour pattern has been, until

recently, unknown for any animal. The data on this subject for man, the most studied animal in this regard, are still inconclusive (Hudelson, 1928). The effects of numbers present upon the rate of conditioning in simple mazes has recently received some attention in this laboratory, and experiments are still in progress with a number of animals distributed fairly widely in the taxonomic series.

Cockroaches are the simplest animals upon which precise results are available to date. Szymanski (1912) and Turner (1912, 1913) demonstrated that these generalised insects could be conditioned on a simple maze. In our experiments with these insects (*Periplaneta americana*), the animals were tested on the open maze shown in Fig. 3, which consisted of a platform with three runways mounted over a pan of water, with the reward, a dark bottle, always at the end of the middle runway. In general these experiments (Gates and Allee, 1933) showed that not all the cockroaches tested could be conditioned to run such a maze, even when capable of taking simpler training. We found no evidence of carrying over of maze

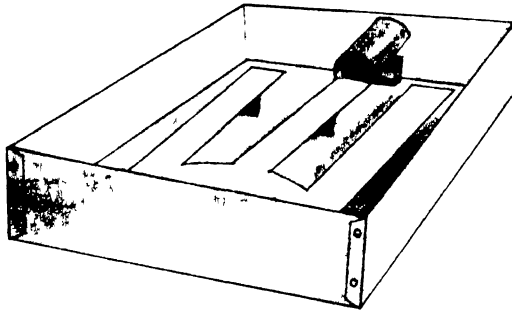


Fig. 3 The maze used in training cockroaches (from Gates and Allee, 1933).

conditioning from day to day, but fifteen to twenty-five successive trials on a given day did yield distinct learning curves of the saw-toothed variety. Similar curves were obtained for time per trial and error per trial.

A cockroach, when isolated, can be conditioned with less time and fewer errors per trial than when the same cockroach is a member of a pair or of a group of three. In the paired and grouped condition, activity is reduced and accordingly the number of errors per minute is also reduced, while the number of errors per trial is increased. The difference in time per trial found in one lot of cockroaches when isolated, paired and members of a group of three is shown in Fig. 4.

A similar group retarding of conditioning is shown when mudminnows (*Umbra limi*) are being trained to jump out of water when a red light is turned on and seize a bit of earthworm loosely impaled on a wire (Welty, 1934). A part of this retardation is due to the lessened opportunity of jumping at a given time; two fishes rarely jump together, and three never did in the course of these experiments. There was a definite group antagonism which prevented jumps otherwise possible. As one fish would move up and assume the jumping attitude another would often attack it with a vigorous jab in the belly and no jump would occur.

Opposed to these two cases in which the group retarded the rate of conditioning there is to be set the extensive experience of Welty, mainly with the common goldfish (*Carassius auratus*), in simple mazes in which the grouped fishes became conditioned consistently more rapidly than did the isolated ones. One of the simpler mazes used is shown in the larger side of the aquarium sketched in Fig. 5. It consisted of a rectangular aquarium 12 in. long with a transverse partition of coarse wire screen located near the forward, more exposed end. The gateway was placed in later experiments at one side at the bottom and was closed between trials by a gate made of a heavy aeroplane celluloid. The fish or fishes to be tested were

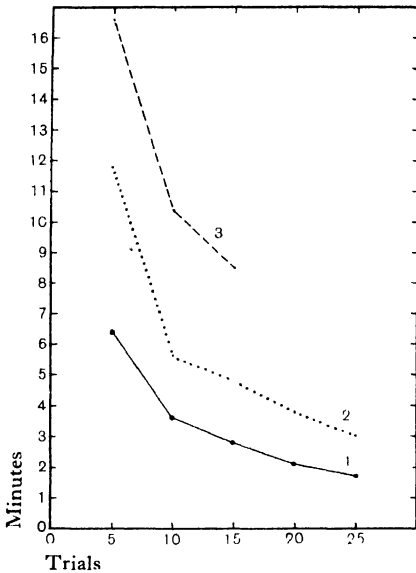


Fig. 4.

Fig. 4. Average time in minutes per trial of all the roaches used in series II, summarised in successive sets of five trials; the isolated animals (curve 1) reacted most rapidly (data from Gates and Allee, 1933).

Fig. 5. The aquarium-maze used in training fishes (from Welty, 1934).

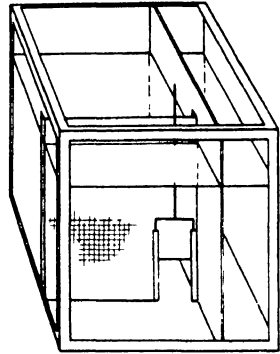


Fig. 5.

introduced when the door was open and were allowed to remain for an acclimatisation period of some days. They were then restricted to the larger division of the aquarium except at the time of the daily test, at which time the lighting of the aquarium was increased either by using an artificial light or by removing a shading cardboard from the free end of the aquarium. The gate was immediately lifted and the time taken with a stopwatch for each fish to move into the forward compartment. Each fish was fed with a bit of worm just after it came through the opening. When more than one fish was present, each was appropriately marked. A number of similar aquarium-mazes were placed side by side with the sides and rear end covered by black paper to secure greater isolation.

In these experiments 928 fishes were used. After once being conditioned they were permanently discarded except as noted on p. 37. In a typical experiment, the

results of which are shown graphically in Fig. 6, there were eight fishes isolated into separate mazes; eight more divided into groups of two and placed in four mazes; eight more divided into groups of four and placed in two mazes; and finally eight more placed all together in one aquarium-maze. As shown by the graphs, the results of this experiment reveal a pronounced group effect; in general, the learning curves follow the size of grouping; the larger the group, the quicker the conditioning.

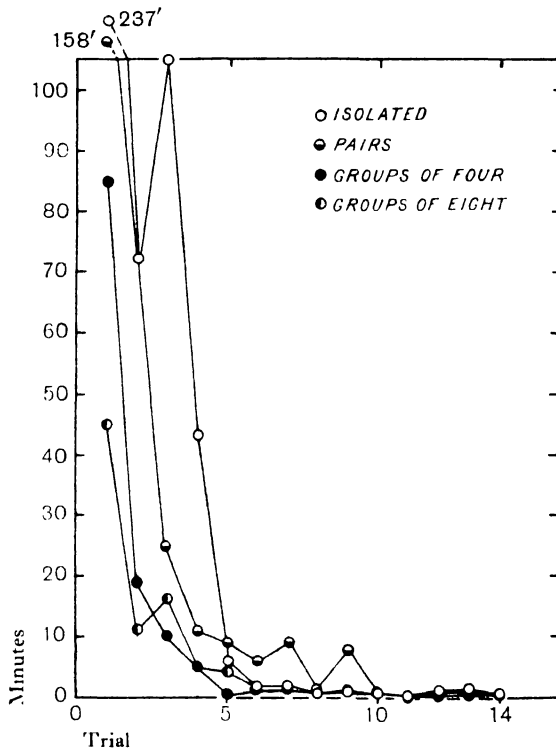


Fig. 6. Mean reaction time for goldfishes in a simple aquarium-maze showing influence of different numbers present (from Welty, 1934)

When untrained goldfishes were introduced into such a simple maze together with a trained goldfish of approximately the same size, the rate of conditioning was increased as compared with a group of the same number of previously untrained goldfishes (see Fig. 7). The presence of such a trained leader contributed in two ways to the more rapid conditioning of his mates: first, he moved through the opening from the larger (rear) division to the smaller feeding area, and, second, he tended to remain in the feeding area, thus acting as a lure to the more slowly reacting untrained fishes. Appropriate experiments indicate that the movement through the opening is more important than the so-called lure effect in conditioning the associated fishes.

There was also other evidence of the existence of group cohesion. The group

tends to retard the first individual exploration of the gateway and feeding chamber, but on the other hand the last fish of the group tends to react more quickly than does the last of a similar number of isolated fishes. Further indication of group cohesion was gained by training small lots of a marine fish (*Cyprinodon variegatus*) as follows: The fishes were placed in the central region of a large aquarium which had narrow feeding spaces screened off at both the rear and the forward ends. These were connected with the main body of the aquarium by the usual simple gate-closed openings. The four fishes in aquarium 1 were trained as usual to come through the forward door and be fed in the front feeding chamber. In aquarium 2, marked fishes *A* and *B* were similarly trained, while *C* and *D* were fed only when they entered the rear feeding place. Aquarium 3 contained four fishes which were trained to pass into the rear feeding chamber, and the fourth and last lot of the series were treated as in aquarium 2.

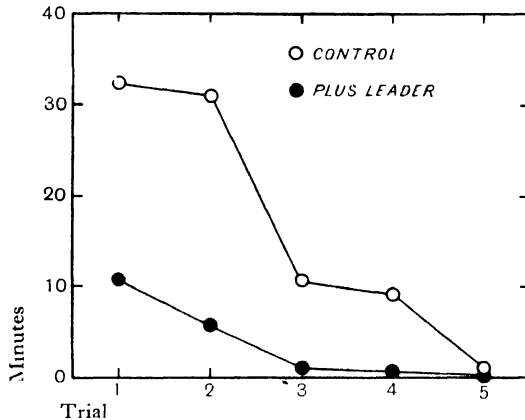


Fig. 7. Mean reaction time of groups of goldfishes in a simple aquarium-maze showing influence of trained leadership (from Welty, 1934).

These experiments showed clearly that the cohering groups were conditioned more rapidly than were the separating groups. The data for the marine *Fundulus heteroclitus* and for the fresh-water goldfishes were less conclusive, but gave similar indications of group cohesion.

Since the experiments indicated that the group effect shown in these maze behaviour experiments was due to visual stimuli the analysis was pushed a step further. The aquarium shown in Fig. 6 was prepared so that at one side of the simple aquarium-maze there was a narrow space separated from the larger portion by a water-tight partition. In half of these aquaria this was of clear glass, in the other half, of opaque. Fishes were present in these side spaces, while previously trained fishes performed in the accompanying mazes. After some days the trained fishes were removed and those from the side spaces were changed into the aquarium-maze. A short interval was given for recovery from the shock produced by handling and then the training of these transferred fishes was begun. In each case those separated by clear glass which allowed ready vision of the behaviour of the fishes

undergoing training showed a more rapid conditioning on being transferred to the maze than did those separated by opaque glass.

Without assuming imitation, as usually defined, these results can be explained on the basis that the fishes behind the transparent glass became "reassured" by the lack of flight behaviour in the fishes undergoing training and on this account were more quiet when transferred to the aquarium-maze. Such "reassurance" was impossible for the fishes behind opaque glass. Also, the fishes behind the clear glass partitions exhibited their group cohesion by moving forward with the fishes under training as these moved into the feeding portion of the aquarium-maze; with these fishes the increase in illumination came to be followed by a forward movement, which was kept up after their transfer to the maze portion of the aquarium. Such a reaction could not be conditioned in this manner by the fishes behind the opaque screen.

The accelerated rate of learning of the group depends in part on group cohesion and in part on two other important factors. Observations, such as those on the rate of feeding of grouped and isolated fishes (p. 7), demonstrate that inter-stimulation between members of a group kept the grouped fishes active at times when many of the isolated animals were inactive; the greater the normal, unhurried activity of a fish, particularly in early exposures in the maze, the greater the chance of finding and passing through the gateway and receiving food. Paradoxically enough, the other group effect which makes for more rapid learning of the simple maze is a quieting effect of the group. Fright reactions retard learning in fishes, and isolated animals were more likely to show them; the normal, relatively quiet, exploratory movements are more nearly characteristic of grouped than of isolated goldfishes.

It is unsafe to predict, on the basis of these experiments upon the effect of grouping upon the rate of learning in fishes, the effect of grouping on the rate of learning in general in other animals. It is particularly unsafe to attempt to apply these results directly to the problem of the effect of class size upon the rate of learning in man. The only safe generalisation that can be drawn is that the nature of the group effect, even in fishes, depends in part on experimental conditions. Presumably, when the returns are all in, a similar relation will be found to hold with regard to the effect of group size upon the rate of learning in man.

XIV. GROUP ORGANISATION.

Schjelderup-Ebbe (1922) has analysed the organisation of flocks of domestic chickens and of both tame and wild ducks (1923). In these he found a more or less definite organisation revealed by the way in which the birds react in contact situations. He recognised a certain peck-order, in which the animals high in the order peck and are not pecked in return, while those at the bottom of the order are pecked without pecking in return.

More recently (1931) Schjelderup-Ebbe has extended his observations to include a large number of different sorts of birds, both in nature and in various types of confinement; he finds that when two birds of one species are together, one is

despot and the other is subservient. Schjelderup-Ebbe believes that despotism is one of the fundamental principles of biology.

The oldest bird of the flock is usually despot because her matured body gives her strength which the young, partially developed birds lack; even after the latter attain full size and strength, if of the same sex the older individual maintains her despotic rights. Between the sexes, the larger males are usually despots over the females. When the two sexes are alike in size and strength and the male possesses ornamentation he is despot; otherwise either may be despot. Often males put on and lose their despotic rights with the assumption and loss of breeding plumage.

We have repeated Schjelderup-Ebbe's observations first on the domestic fowl, both males and females, and then with flocks of pigeons, both mated and with sexes segregated (Masur and Allee, in press). These observations have confirmed, in general, his original conclusions, at least in so far as chickens are concerned. With pigeons, in place of an absolute peck-right in which one of any given contact-pair is always dominant and the other always subservient, we found a relationship which may be called "peck-dominance." This means that while one member of a contact-pair usually dominates in its contacts with the other, the relationship is by no means fixed and, in fact, may change within an hour's time. Such reversals are not permanent, as is shown by the fact that in the long run one bird usually dominates oftener than it is subordinate to the other of a contact-pair. The occurrence of such reversals seems to be a fixed part of the social organisation of the pigeon flock we investigated. We also found with some of the pigeons that there is a relation between spatial position and dominance. Thus one female tended to be dominant when at the entrance of the cote while another was dominant when on the ground. Even with the pigeons, however, the flock was fairly definitely organised with certain individuals usually showing peck-dominance and certain others being usually subservient.

It is not a far cry from such studies of the internal organisation of bird flocks to the questions centring about flock leadership. One of the most striking of these problems is illustrated by the way in which bird flocks, particularly flocks of shore birds, fly at times in close formation and seemingly instantaneously wheel in unison as though each individual were simultaneously motivated by a common impulse rather than adjusting itself to the movements of the other members of the flock. Nichols (1931) reports pertinent observations on this point. Two young Dowitchers were discovered flocking temporarily with a dozen black-bellied plovers and a single golden plover. When flushed, the flight of the latter was comparatively rapid and it was soon ahead of the flock; as the latter wheeled, the golden plover, finding itself alone, rose above the flock and dived down ahead with a few swift wing beats. The Dowitchers, slowest in flight of all in the mixed group, took the chord of the arc of the wheeling flock, caught up and again became an integral part.

These observations do not reveal the stimulus which releases the wheeling mechanism of the main flock. The simplest explanation, that the leader, finding himself out alone in front, starts to turn and so gives a stimulus to the remainder

of the flock, does not seem to hold when it is tested by observations with flocks of wheeling pigeons. In such flocks the stimulus to turn frequently originates in the lateral front rank. Such a change is correlated with a change in the immediate leaders. The apparent leaders may not be the actual ones, for the faster individuals may drive through the flock to the foremost place, taking their direction from the flock, rather than *vice versa*.

The similarity of organisation of bird flocks to other groups, including those of man, is close enough to be at least amusing, and the similarity of the pseudo-leadership shown by the golden plover to some varieties of human leadership does not need to be stressed. Students concerned with problems of leadership in human affairs would do well to devote serious study to the types of leadership to be found among other animals.

This conclusion is strengthened by the recent studies on the group organisation and behaviour of monkeys and apes. Fortunately some of these observations have been made in nature, and while lacking in the intimate detail of observation on animals caged or otherwise confined, they inspire more confidence that the normal behaviour is being recorded, rather than behaviour possibilities under artificial conditions. Zuckerman's (1932) excellent observations on the sexual life of baboons suffer in this respect, in the same way that observation of human behaviour in prison, even on modern prison farms, would hardly be a fair basis for the interpretation of human behaviour in general, although containing many elements common with human behaviour under more normal conditions. Zuckerman records seeing repeated in the field many of the details of behaviour shown by baboons in loose confinement, but one cannot be sure that their preoccupation with sexual affairs, for example, would be as intense in the field as in confinement. From Zuckerman's observations it appears that the baboon horde is organised into smaller groups consisting of a dominant male, one or more females and their young, and one or more "bachelor" hangers-on.

From observations in the field, Nissen (1931) reports that chimpanzees also are organised in groups. One animal, in five instances identified as a male, stood out from the rest of the group by his superior size. In five cases the largest animal was recognised as a leader when a general movement of the band was in progress; in one instance this large leader was known to be a female. While certain individuals have precedence over others, largely as a matter of size, evidence is lacking that the group is dominated by a despotic leader. The large amount of vocalisation in these bands invites further study, particularly in view of the tendency among some students of social behaviour of man (Rabaud, 1931) to emphasise the uniqueness of man as a "language animal."

XV. SUMMARY.

The present review of the literature of mass physiology is limited to the years just previous to 1933, and for approximate completeness must be read in connection with an earlier and more extensive survey (Allee, 1931). Analysis of the

reactions leading to the formation of aggregations in nature or in the laboratory has scarcely proceeded beyond the recognition that much of such behaviour is innate. There is, however, recent evidence that a part of the schooling behaviour of the fish *Ameiurus* is acquired rather than inherited.

Once formed, aggregations of aquatic organisms condition the medium surrounding them by the addition of secretions and excretions, the nature and the biological effects of which form one of the important problems of mass physiology.

It is easy to demonstrate that overcrowding lessens the rate of growth of organisms. More recently evidence has been accumulating that undercrowding frequently has the same effect. Evidence is presented on this point in such widely different animals as mealworms, fishes and mice. Similarly, with population growth the harmful effects of undercrowding have recently been found for protozoans, crustaceans and beetles, as well as the ill effects of overcrowding.

The results from aggregation upon rate of oxygen consumption varies with different animals, and even with the same animals at different times of the year. With goldfishes, those in small groups use less oxygen per individual if grouped than when isolated; with the more closely schooling *Ameiurus* opposite results are reported. Outside the breeding season, the brittle starfish *Ophioderma* consumes less oxygen per individual if grouped; this relationship does not necessarily hold during the breeding season.

Groups of animals are able to afford protection to their members if exposed to toxic conditions produced either by the absence of accustomed salts, as the marine flatworm *Procerodes* does when placed in fresh water, or by the presence of toxic substances such as colloidal silver. The amount of protection furnished has been measured for certain cases, and the protective mechanisms are discussed.

The polarity of the seaweed *Fucus* can be determined by the position of a given egg with reference to a group of other eggs of the same or of a different species. Such plasticity might be expected from plants more readily than from animals. With animals, Uvarov's phase theory of locusts has been experimentally demonstrated to hold for the South African *Locustana pardalina*. The transition from parthenogenetic to sexual reproduction in certain cladocerans has again been demonstrated to result from crowding, and considerable progress has been made in the analysis of the relative importance of the presence of metabolic products and of nutrition in the control of sex in these animals.

The effects of numbers present upon the rate of learning differs with different animals and even in the same animals with different problems. Thus fishes learn to run a simple maze more rapidly if in groups than if isolated, but they learn less readily to jump for a bit of worm held just above the water level. Cockroaches also learn to run a simple maze more slowly if more than one is present in the maze at the same time.

Groups of birds show a fairly definite flock organisation, which may or may not be related to active leadership of the flock.

The whole range of mass physiology has been presented with the thought that it forms a large part of the background for social life.

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¹ Skeleton citations without title are duplications of the bibliography in Allee, *Animal Aggregations*, 1931. In order to present a more complete bibliography, some titles are included which are not reviewed in the text.

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DIE SICHTBARKEIT ULTRAVIOLETTEN LICHTES

VON E. MERKER.

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INHALTSÜBERSICHT.

	SEITE
I. Einleitung	49
II. Die Leistung der Wirbeltieraugen in ultraviolettem Licht	52
(1) Die Fluoreszenz der Gewebe in Wirbeltieraugen	52
(2) Die Durchlässigkeit der Gewebe von Wirbeltieraugen für ultraviolettes Licht	54
(3) Die physiologische Wirksamkeit des ultravioletten Lichtes und des Fluoreszenzlichtes im Auge	57
(4) Die Schädigung des Wirbeltierauges durch Ultraviolett	60
III. Die Bewegungen von Pigment und von Stäbchen und Zapfen in der Netzhaut des Fisches unter dem Einfluss von ultraviolettem Licht	61
IV. Die Pigmentbewegung im Superpositionsauge von Insekten unter dem Einfluss von ultraviolettem Licht	64
(1) Das Fluoreszenzlicht im Insektenauge	64
(2) Ultraviolettes Licht wird trotz der Chitinfluoreszenz und der Fluoreszenz der Gewebe auf dem Grunde der Netzaugen von Insekten nachgewiesen	65
V. Die Ausdehnung des sichtbaren Spektrums für <i>Drosophila</i> und die Honigbiene sowie die Verteilung der bewegungsanregenden Kraft in diesem Spektrum	67
VI. Die Daphnien sehen ultraviolettes Licht	69
VII. Das Sehvermögen der Planarien in ultraviolettem Licht	71
VIII. Zusammenfassung	75
IX. Summary	76
Acknowledgments	77
Literaturverzeichnis	77

I. EINLEITUNG.

WIR können bisher nur von wenigen Lebewesen mit Sicherheit sagen, dass sie ultraviolettes Licht von anderem Licht zu unterscheiden vermögen, dass sie ultraviolettes Licht sehen. Doch ist auf Grund unserer Erfahrungen zu erwarten, dass es auch noch von vielen anderen gesehen wird. Insbesondere dürfte dies für das violett-nahe Ultraviolett gelten. Jedoch muss es für jeden einzelnen Fall festgestellt werden. Der Beweis hierfür ist nicht ganz leicht zu erbringen. Das wird aus Folgendem verständlich werden.

Zu den Wesen, die sicher das Ultraviolett erkennen, gehört ausser dem Menschen, der Wasserschnecke (*Daphnia pulex*), gehören die Süßwasserplanarien, die Bienen und *Drosophila* und unter den Wirbeltieren höchstwahrscheinlich der Stichling und der Frosch: *Rana temporaria* entwickelt bei Belichtung mit langwelligem Ultraviolett Netzhautströme (Merker, 1933).

Es ist eine wohlbekannte Tatsache, dass wir Menschen ultraviolettes Licht erkennen. Sie geht auf Soret (1883) zurück und lässt sich, trotz häufiger gegenteiliger Ansicht nur immer wieder bestätigen. Nach Soret sehen wir das Ultraviolett nur bis zur Wellenlänge $\lambda = 330 \text{ m}\mu$. Diese Grenze liegt nach neueren Untersuchungen nicht unbedingt fest. Sie kann je nach Beschaffenheit der Augen weiter in das kurzwellige Gebiet wie auch nach dem violetten Bereich hin verschoben sein. Im Alter sinkt die Durchlässigkeit, während nach Krankheit (Hallauer, 1909 b) die Augenbestandteile durchlässiger für die kurzwelligen Strahlen geworden sein können. Dass hier überhaupt eine Absorptionsgrenze zu finden ist, hängt mit der starken Absorption der kurzwelligen Strahlen in der Augenoptik, hauptsächlich der Linse, zusammen. Die Eiweisskörper, woraus sich diese Organe aufbauen, sind im Wesentlichen an dieser Absorption schuld. Es ist durchaus nicht richtig anzunehmen, dass unsere Netzhaut als Empfänger den kurzwelligen Strahlen gegenüber versagt. Man findet im Gegenteil berichtet, dass nach Staroperationen Lichteindrücke auch noch von viel kurzwelligerem Licht ($\lambda = 250 \text{ m}\mu$) empfunden werden.

Für gewöhnlich sehen wir Menschen vom Ultraviolett des Tageslichtes gar nichts. Doch braucht uns dieser Umstand nicht mehr zu wundern als die Tatsache, dass uns das Blau oder das Rot aus dem Himmelslicht nicht gesondert entgegentritt. Bedeutungsvoller ist jedoch, dass wir keine Farbe namens "Ultraviolett" kennen.

Das Unterscheidungsvermögen der *Daphnia pulex* für ultraviolettes Licht ist durch neue Versuche bestätigt worden (Merker, 1930). Die grundlegenden Arbeiten von S. Becher (1921, 1923) haben gezeigt, dass die Daphnien noch auf sehr kurzwelliges Licht ($\lambda = 253 \text{ m}\mu$) ansprechen. Doch war die Wirksamkeit der Fluoreszenz, die in den Rhabdomen im ultravioletten Licht entsteht, nicht ausreichend geklärt. Durch die späteren Untersuchungen wurde die Fluoreszenzwirkung in den Augen geprüft und als schwach befunden. Das ultraviolette Licht übertönt sie. Bisher gilt dies nur für das langwellige Ultraviolett. Wir vermuten jedoch wegen der hohen Durchlässigkeit des ganzen Körpers für das gesamte Spektrum der Quarzlampe und im Hinblick auf die Ergebnisse von Becher, dass auch das kurzwellige Ultraviolett gesehen wird.

Für die Süsswasserplanarien gilt Ähnliches (Merker, 1932). Sie sehen bestimmt das langwellige Ultraviolett. Von einigen wissen wir bereits, dass sie z.B. auch auf das Licht der Linien $\lambda = 313-296 \text{ m}\mu$ antworten, während *Dendrocoelum lacteum* dem Licht bis zur Linie $\lambda = 253 \text{ m}\mu$ ausweicht. Alle früheren Angaben über das Sehvermögen von Fischen und Krebsen im Ultraviolett (Schiemenz, 1924; Wolff, 1925) enthalten wohl beachtenswerte Hinweise, doch sind sie nicht bindend, obwohl sie vielfach benutzt und verbreitet worden sind. Sie müssen deshalb nachgeprüft werden, weil die Fluoreszenzhelligkeit der Gewebe bei jenen Untersuchungen nicht gegen die Ultraviolett-Wirkung abgewogen wurden. Schon v. Hess (1911) hatte auf die Fluoreszenz der brechenden Augenbestandteile bei Insekten hingewiesen und insbesondere auch die Fluoreszenz des Chitins erkannt. Er gründete darauf, die heute ebenfalls unhaltbare Ansicht, dass alles Ultraviolett als sichtbares

Licht wirke, weil es durch das fluoreszierende Leuchten der Augenoptik in sichtbares Licht verwandelt würde.

Die Beobachtung von v. Hess war an sich richtig, nicht aber seine Schlüsse. Heute wissen wir, dass alles farblose Gewebe im Ultraviolett fluoresziert und dass die Fluoreszenz recht erheblich werden kann, wenn z.B. die Augen Rückstrahlerschichten (Tapeta) ausgebildet haben (Merker, 1928, 1929 b). Dass die Augengewebe der Wirbeltiere, insbesondere ihre Linsen stark fluoreszieren, war schon v. Helmholtz bekannt. "Sie strahlen dabei weissblaues Licht aus, ähnlich der Chitinlösungen," schreibt er (1911, vol. II, p. 60). Neuerdings hat sich nun herausgestellt, dass die Fluoreszenz im Insektenauge, obwohl sie vielfach festgestellt wurde, aus bestimmten Gründen keine grosse Rolle spielt. Die Empfindlichkeit für Ultraviolett ist nämlich erstaunlich hoch und übertrifft die Empfindlichkeit für sichtbares Licht um ein Mehrfaches. Diese Feststellungen sind Bertholf gelungen. Er hat an Bienen (1931) und *Drosophila* (1932) die bewegungsanregende Kraft der einzelnen Ultraviolett-Linie des Hg-Spektrums mit einer ausgemessenen Weisselligkeit verglichen.

Für beide Tiere fand er bei $\lambda = 365 \text{ m}\mu$ ein ungemein hohes Maximum. Insbesondere sind die letzteren Untersuchungen auch technisch zuverlässiger geworden, sodass es feststeht, dass Bienen und Taufiegen ultraviolettes Licht sehen. Von den Bienen hat man es längst geglaubt (Kühn, 1924, 1927). Die Verhältnisse liegen ähnlich wie bei uns selbst, den Daphnien und den Planarien, wenn auch die Empfindlichkeiten im Ultraviolett für die verschiedenen Tierarten recht verschieden sein mögen.

Unter ultraviolettem Licht verstehen wir den Wellenbereich im Spektrum, der sich jenseits der Wellenlänge von $\lambda = 400 \text{ m}\mu$ etwa bis zur Wellenlänge von $200 \text{ m}\mu$ erstreckt. Die Wellenlängen von $\lambda = 400 \text{ m}\mu$ bis $300 \text{ m}\mu$ finden sich auch im Tageslicht. Wir nennen deshalb diesen Bezirk das Sonnenultraviolett. Der Wellenbereich von $\lambda = 300 \text{ m}\mu$ bis $200 \text{ m}\mu$ kann nur durch besondere Geräte im Laboratorium erzeugt werden (Quarzlampe).

Bei unseren Versuchen lieferte uns gewöhnlich die Quarzlampe das ultraviolette Licht. Sie gestattet ein Linienspektrum zu entwerfen, das bequem das Herausblenden einzelner Linien in völliger Reinheit zulässt.

Unter dem Sammelnamen Ultraviolett vereinigen wir also höchst verschiedenwelliges und verschiedenartiges Licht. Es ist daher kaum zu erwarten, dass im gesamten ultravioletten Gebiet die physiologischen Wirkungen, die uns hier angehen, gleichartig sind.

So wird man denn zunächst festzustellen haben, ob die Augenbestandteile der verschiedenen Tiere überhaupt für die ultravioletten Strahlen alle durchlässig sind. Wenn das nicht zutreffen sollte, so wäre nachzuforschen, für welche Augenteile und für welche Strahlen Unterschiede bestehen und wie sie sich auswirken. Wissen wir doch, dass gar viele organische Stoffe im ultravioletten Licht fluoreszieren, d.h. ultraviolettes Licht verschlucken und dafür Licht aus den sichtbaren Bezirken des Spektrums abgeben.

Mit dem Erscheinen von *Fluoreszenz* ist ein Verlust von ultraviolettem Licht

verbunden. Wo sie sich zeigt, muss man nachsehen, wie hoch die Absorption ist. Aber nicht alles absorbierte Licht erscheint in anderer Wellenlänge wieder, wird Fluoreszenzlicht. Ein Teil des eingestrahnten Lichtes wird verschluckt, ohne wieder sichtbar zu werden. Ein anderer Teil wird unverändert durchgelassen. Wie hoch er ist, kann bisher nur für die Rindsaugen gesagt werden (Roggenbau und Wettbauer, 1927). Die stets auftretende Fluoreszenz macht jeden Ultraviolettversuch unrein. Das sichtbare Fluoreszenzlicht mischt sich in das kürzerwellige Ultraviolett hinein. Bei Lichtsinnesversuchen ist das besonders störend. Darum muss man stets den Wirkungsgrad des Fluoreszenzlichtes bestimmen.

II. DIE LEISTUNG DER WIRBELTIERAUGEN IN ULTRAVIOLETTEM LICHT.

Wir haben die Wirksamkeit des ultravioletten Lichtes und die Wirksamkeit des gleichzeitig durch das Ultraviolett entstehenden Fluoreszenzlichtes zunächst an Wirbeltieraugen nachgeprüft. Untersucht haben wir die leicht erreichbaren Säuger- und Vogeltieraugen, Reptilien- und Amphibienaugen. Im Wesentlichen zeigten sich bei allen ähnliche Erscheinungen.

(1) *Die Fluoreszenz der Gewebe in Wirbeltieraugen.*

Die Fluoreszenzen, die an den Wirbeltieraugen äusserlich im reinen Ultraviolett auftreten, sind recht auffallend. Die Augen erscheinen völlig verändert. Das Weisse des Augapfels leuchtet sehr stark. Die Linse füllt grau das Sehloch aus wie bei Totenagen.

Auch im Innern der Augen ist Fluoreszenz zu beobachten. Tatsächlich leuchtet alles farblose Gewebe. Wo das Gewebe getönt oder pigmentiert ist, erscheint die Fluoreszenz entweder schwächer, im Ton verändert oder überhaupt aufgehoben. Das Fluoreszenzlicht der farblosen Gewebe ist sehr einheitlich zusammengesetzt. Es kann vom Rot bis zum Violett alles Licht des sichtbaren Spektrums vertreten sein, doch in anderen Anteilen als im Tageslicht. Daher ist auch die Farbe dieses Fluoreszenzlichtes blauweiss und nicht gelblichweiss wie das Tageslicht.

Wenn man den Gefrierschnitt durch ein Wirbeltierauge im ultravioletten Licht unter dem Mikroskop betrachtet, so erkennt man deutlich, dass das farblose Gewebe leuchtet. Cornea, Linse, auch Glaskörper und Netzhaut leuchten, wo kein Pigment sitzt. Die harte Augenhaut um das Auge herum leuchtet ebenfalls wie alles Bindegewebe recht erheblich. Die Linsen strahlen ganz besonders stark. Herausgenommene Linsen aus Rindsaugen, Bussardaugen oder Froschaugen leuchten im dunklen Ultraviolett bläulichweiss wie Edelsteine.

In der Stärke der Linsenfluoreszenz haben wir sehr grosse Unterschiede gefunden. Die hier folgende Zusammenstellung gibt darüber Aufschlüsse.

Stark leuchtende Linsen	Schwächer leuchtende Linsen
Frösche, Molche Wildente, Bussard Rind, Schaf, Schwein	Kröten, Alpensalamander Schleiereule, Waldkauz Katzen, Fledermaus, Waldmaus

Auf Grund dieser Erfahrungen kann man auf den Gedanken kommen, dass Schattentiere und Dämmerungstiere vielleicht durchweg nicht so stark leuchtende Augenlinsen besitzen, wie die Tagestiere (Merker, 1928). Auch Shoji (1922) steht dieser Auffassung nicht fern. Er fand bereits vor uns, dass die Augenteile der Eulen sehr grosse Durchlässigkeit für ultraviolettes Licht besitzen.

Jungtiere haben ganz deutlich weniger stark leuchtende Linsen als ältere Tiere. Umgekehrt verhält sich ihre Durchlässigkeit. Von Rinderlinsen ist das schon seit Soret (1883), De Chardonnet (1883), Birch-Hirschfeld (1909) bekannt und neuerdings von Shoji (1922) und zahlenmässig von Roggenbau und Wetthauer (1927) bestätigt worden. An Menschenlinsen hat es Hallauer (1909 b) gesehen.

Das Austrocknen der Linsen oder Wasserentzug durch Alkohol führt zu sehr erheblicher Steigerung der Fluoreszenz. Die Linsen von Jungtieren, die noch ganz besonders dilut leuchtend sind, zeigen nach dem Trocknen ein kalkiges Leuchten. Umgekehrt wird durch Auflösen der Linsen, entsprechend der Verdünnung, das Leuchten matter und grau.

Das deutet wohl darauf hin, dass mit einer Verhärtung im Alter die Fluoreszenz der Augenlinse zunimmt. Vielleicht spielt auch das Licht selbst, je länger es die Linse durchstrahlt, eine fluoreszenzerhöhende Rolle. Die Untersuchungen von Wels (1928) und Wels und Jokisch (1930) über die Verstärkung der Fluoreszenz von Zellen und Geweben unter dem Einfluss von ultraviolettem Licht deuten auf eine solche Möglichkeit. Die Unterschiede zwischen Hell- und Dunkeltieren sind vielleicht so entstanden zu denken. Vielleicht verändern auch beide Faktoren zusammen die Augenlinsen so, dass sie, je länger, desto mehr, im Ultraviolett leuchten.

Die klare Hornhaut eines Froschauges ist nach einer Bestrahlung von einer Stunde mit dem vollen Licht der Quarzlampe deutlich getrübt. Beim Vorschalten einer aufliegenden Stannioblende wird die Grenze der beleuchteten Fläche scharf sichtbar. Die feuchte Haut riecht verbrannt wie unsere eigne Haut nach Ultraviolettbestrahlung zu riechen pflegt.

Die Fluoreszenz einer Augenlinse kann bis auf den Kern in der Mitte zum Verschwinden gebracht werden, wenn man sie mit verdünntem Glycerin tränkt. Ersatz des Glycerins durch Wasser stellt die alte Fluoreszenz z.T. wieder her. Jegliche Färbung der Linse beeinträchtigt die Fluoreszenzhelligkeit. Die Augenlinsen von Eichhörnchen sind schon in früher Jugend gelb gefärbt, was sich im Alter noch steigert. Wie bei altersgelben Menschenlinsen ist auch hier die Fluoreszenz im *Ultraviolett* stark gemindert und in der Farbe statt weisslichblau gelblichgrüngrau. In Glycerin zieht die gelbe Farbe der Linse des Eichhörnchens aus, und die gewöhnliche weisslichblaue Fluoreszenz kommt zum Vorschein.

Die Gelbfärbung der Linse führt zur Herabsetzung der Fluoreszenz, zugleich aber auch zur Herabminderung der Lichtdurchlässigkeit. Zusatz von verdünntem Glycerin dämpft zwar auch die Stärke der Fluoreszenz, jedoch im Sinne einer Homogenisierung, denn die Durchlässigkeit für Licht wird grösser.

(2) *Die Durchlässigkeit der Gewebe von Wirbeltieraugen
für ultraviolettes Licht.*

Angesichts der Tatsache, dass durch die Fluoreszenz der Gewebe in den Augen von dem eingestrahnten Ultraviolett ein Anteil verschluckt wird, ist die Frage erlaubt, wieviel Ultraviolett überhaupt auf die Netzhaut gelangt. Man wird also die Durchlässigkeit der Augenteile für ultraviolettes Licht zu prüfen haben. Wir erhalten über diese Durchlässigkeit durch Spektrogramme am besten einen Überblick, die die Restspektren zeigen, nachdem das Licht der Quarzlampe durch die verschiedenen Augenteile gegangen ist. Man erkennt, das z.B. beim Rindsauge jeder Teil für sich, also Cornea, Linse, Glaskörper und Netzhaut ungefähr gleichartig um die Wellenlänge von $\lambda = 300 \text{ m}\mu$ vollständig das kurzwellige Ultraviolett wegfangen. Auch bei Dauerbelichtung kann kein Licht unterhalb der Wellenlänge von $\lambda = 300 \text{ m}\mu$ mehr auf die Netzhaut gelangen, selbst wenn es im Tageslicht ausnahmsweise vorhanden sein sollte.

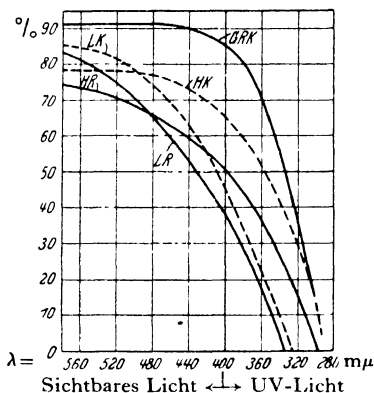


Abb. 1. Die Durchlässigkeit der Augenbestandteile des Rindes und des Kalbes für sichtbares und ultraviolettes Licht in Prozenten der auftreffenden Energien. (Aus Roggenbau und Wetthauer, 1927, S. 768.) Starke Absorption im Ultraviolett nach dem Überschreiten der Grenze des sogenannten sichtbaren Lichtes ($\lambda = 400 \text{ m}\mu$). GRK = Glaskörper vom Rind und Kalb; beide zeigen gleiche Absorption. LR = Linse vom Rindsauge. LK = Linse vom Kalbsauge. HR = Hornhaut vom Rindsauge.

Diese Tatsache war schon v. Helmholtz (1911) bekannt und wurde später noch öfter bestätigt (Shoji, 1922; Roggenbau und Wetthauer, 1927). Genau genommen absorbiert die Linse am stärksten die ultravioletten Strahlen. Es folgen der Reihe nach Hornhaut, Glaskörper und das Kammerwasser.

Allein die Netzhaut zeigt auf unserm Bild noch unterhalb der Wellenlänge von $\lambda = 300 \text{ m}\mu$ schwache Durchlässigkeit. Nach den Angaben von Shoji ist sie noch weit durchlässiger als unsere Aufnahme verrät. Das mag individuell verschieden sein.

Roggenbau und Wetthauer haben als die Ersten den Grad der Absorption für die verschiedenen Wellenlängen des Quarzquecksilberlichtes photogramm-metrisch bestimmt. Sie untersuchten die grossen und für diese Zwecke sehr geeigneten

Kalbs- und Rindsaugen und bestätigten zahlenmässig die oft gesehene Tatsache, dass jugendliche Augen lichtdurchlässiger sind als ältere Augen.

Auf der Abb. 1 sind die Absorptionskurven für die verschiedenen Augenteile vom Rind und Kalb eingetragen, wie sie Roggenbau und Wetthauer (1927) bestimmt haben. Tab. I gibt Durchlässigkeitszahlen in Prozenten der eingestrahnten Lichtenergie.

Tabelle I. Die Durchlässigkeit der Augenteile von Rind und Kalb für sichtbares und für ultraviolettes Licht in Prozenten (nach Angaben von Roggenbau und Wetthauer (1927) zusammengestellt).

Wellenlänge	Rind			Kalb		
	Hornhaut	Linse	Glaskörper	Hornhaut	Linse	Glaskörper
Gelb $\lambda = 578$	73	86	92	77	90	92
Blau $\lambda = 436$	58	52	90	73	63	90
Violett $\lambda = 405$	50	38	85	65	45	85
Ultraviolett $\lambda = 365$	40	20	80	50	25	80
$\lambda = 312$	25	0	63	38	6	63
$\lambda = 303$	3	0	18	15	0	18
$\lambda = 296$	0	0	15	5	0	15

Legt man die obigen Zahlen zugrunde, so erreichen beim Rinde 6.4 Prozent des eingestrahnten Lichtes der Wellenlänge $\lambda = 366 \text{ m}\mu$ die Netzhaut, beim Kalb aber 10 Prozent. Auch erreicht beim Kalb noch kurzwelliges Licht den Augenfundus als beim Rinde. Das Licht der Wellenlänge $\lambda = 312 \text{ m}\mu$ wird im Rindsauge schon vor der Netzhaut völlig verschluckt. Im Kalbsauge dagegen erreicht noch 1 Prozent die Netzhaut.

Das sind sehr geringe Werte, die, aber, wie wir später zeigen werden, doch nicht zu unterschätzen sind. Je kleiner das Auge ist, um so grösser durften diese Zahlen werden. Doch auch die genannte Abhängigkeit gilt nicht unumschränkt, wie uns das Froschauge lehrt.

Immerhin bestätigt es sich, dass ultraviolette Strahlen nur in geringer Menge auf die Netzhaut im Wirbeltierauge gelangen und dass, je kurzwelliger das Licht ist, es um so schwieriger in das Auge einzudringen vermag (Greeff, 1922).

Nicht bei allen Tieren herrschen in den Augen, wie erwähnt, die gleichen *Absorptionsverhältnisse*. Wir konnten es vielfach feststellen. Das Eichhörnchen z.B. hat gelbgefärbte Augenlinsen. Diese Gelbfärbung bedingt eine weit stärkere Absorption als gewöhnlich.

Die Restspektren der Augenteile unserer Frösche (*Rana esculenta* und *R. temporaria*) offenbaren noch andere Unterschiede. Obwohl in unseren Versuchen die Belichtung bei den *Rana*-Spektren länger als bei den Spektren der Augenteile des

Rindes war, schneiden die Froschlinsen das Quecksilberspektrum schärfer und früher ab als die Rindslinsen ($\lambda = 318 \text{ m}\mu$). Die kleine Froschlinse verschluckt das Ultraviolett viel heftiger als die 3–4mal dickere Rindslinse. Die *Dichte* der Linsengewebe spielt also eine Rolle. Wir wissen, dass sie mit dem Alter zunimmt, d.h. dass der Wassergehalt sich verringert, der Eiweissgehalt dagegen relativ und auch absolut zunimmt (Shoji, 1922). Damit aber hängt die Absorptionsfähigkeit der Linsen überhaupt zusammen.

Doch auch artliche Verschiedenheiten in der Dichte der Linsengewebe, in ihrem Eiweissgehalt, müssen wirksam sein. Wir haben hinter Hai-Linsen kein Aufleuchten einer Fluoreszenzplatte mehr gesehen. Sie scheinen für Ultraviolett ziemlich undurchlässig zu sein.

In noch höherem Masse sind (wie erwähnt) die kleinen Froschlinsen undurchlässig. Sie könnten sonst mit ihrer 2.5 mm. Schichtdicke nicht entfernt hinsichtlich der Absorption das leisten, was wir tatsächlich feststellen.

Von den beiden Froscharten hat *Rana temporaria* stets die durchlässigeren Linsen als *R. esculenta*. Doch gilt diese Feststellung nur für die ultravioletten Linien $\lambda = 366 \text{ m}\mu$ bis $\lambda = 313 \text{ m}\mu$. Denn für beide Linsenarten liegt auch bei stundenlanger Belichtung die scharfe Grenze der Durchlässigkeit hinter der Linie $\lambda = 313 \text{ m}\mu$. Sie stimmen hierin mit der Katzenlinse völlig überein. Die Rindslinse aber zeigt auch über diese Grenze hinaus noch Durchlässigkeit. Es mag betont werden, dass sich diese Absorptionsgrenze noch etwas verschieben kann, wenn man linienreicheres Licht (Eisenbogenlicht) durch die Linsen schickt. Man wird einsehen, dass die ganz genaue Absorptionsgrenze vielleicht gerade deshalb nicht verzeichnet werden kann, weil sie in die Linienlücke des Hg-Dampfes von $\lambda = 313 \text{ m}\mu$ bis $\lambda = 304 \text{ m}\mu$ fällt.

Wir haben schon früher darauf aufmerksam gemacht, dass gerade die Esculenta-Linse sehr undurchlässig ist (Merker, 1928). Hier finden wir eine neue Bestätigung dafür. Doch kann man mit der hier verwendeten, empfindlicheren photographischen Methode zeigen, dass bei Dauerbelichtung die Linsen auch für das langwellige Ultraviolett grössere Durchlässigkeit zeigen, als es nach unseren Versuchsergebnissen damals den Anschein hatte. Für die *Sichtbarkeit* von Licht spielt jedoch die Dauerbestrahlung keine Rolle. Die Netzhaut kumuliert die Eindrücke nicht wie die photographische Platte, oder doch nur in ganz beschränktem Masse. Die *Schädigung* durch Licht ist jedoch eine Summenwirkung. Man darf sich also beim Studium der photographischen Spektren durch die Akkumulation der Lichtwirkungen nicht zu falschen Schlüssen verleiten lassen. Für das Sehen spielt lediglich die eingestrahelte Energiehöhe in ganz kurzem Zeitabschnitt eine Rolle.

Die beiden Froschlinsen unterscheiden sich auch in ihrer Fluoreszenzfarbe. Man kann eine Linse von *Rana esculenta* sofort an ihrer gelbgrünen Fluoreszenz erkennen, die jedoch bei den Larven noch nicht vorhanden ist. Die Fluoreszenzfarbe der Larvenlinsen ist wie gewöhnlich bläulichweiss und gleicht der Fluoreszenz der Linsen von *R. temporaria*, die ebenfalls bläulichweiss leuchten. Beim Vergleich der Leistungen von Froschlinsen mit den Linsen vom Rind darf man nicht vergessen, dass die untersuchten Frösche ältere Tiere waren als das untersuchte Rind. Vielleicht ist aber diese Tatsache allein nicht ausreichend, um die Grösse der

Unterschiede verständlich zu machen. An den Spektren der Bestandteile des jungen, noch ungebrauchten Katzenauges fanden wir dass die Linse weniger weit durchlässig ist als die Rindslinse. Sie gleicht in dieser Eigenschaft mehr der Froschlinse, obwohl sie verhältnismässig viel durchlässiger ist. Die Linsenkerne waren bei der jungen Katzenlinse sehr deutlich ausgebildet. Von den Linsenkerne aber wissen wir nach Shoji (1922), dass sie stärker absorbieren, als die übrige Linsensubstanz. Fast sieht es so aus, als ob die embryonalen Augen weniger lichtdurchlässig wären als die Augen in der ersten nachembryonalen Zeit. Hier müssen weitere Untersuchungen aufklären.

Entsprechend der spektrographisch festgestellten Durchlässigkeit der Augenteile im Gebiet des Sonnenultravioletts lassen sich auch deutliche Bilder durch diese Lichtarten auf der Netzhaut der Augen entwerfen. Abb. 2 zeigt die auf dem Augenhintergrunde photographierten ultravioletten *objektiven* Bilder des leuchtenden Quarzbrenners, die durch die Augenoptik erzeugt wurden. Aus dem Licht der Quarzlampe war durch ein Uvetfilter nur die Linien $\lambda = 366 \text{ m}\mu$ und $\lambda = 334 \text{ m}\mu$ ausgeblendet. Es konnte also nur das Licht der eben genannten beiden Linien die Bildchen entwerfen. Die stark photochemische Wirkung des ultravioletten Lichtes liess auf der Platte das Bild des Brenners der Quarzlampe solarisiert erscheinen, auch bei ganz kurzer Belichtungszeit (1–3 Sekunden). Man kann also durch dieses Licht erhebliche Wirkungen auf der Netzhaut erwarten. Indessen sind diese Wirkungen nach unseren Erfahrungen nicht schädlich. Bei einem albinotischen Auge ohne Pigment (weisse Ratte) erzeugt das ultraviolette Licht auf der Netzhaut und den darüberliegenden Schichten ein klares objektives Fluoreszenzbild (Abb. 2 (3)). Dieses Fluoreszenzbild ist grösser als das ultraviolette Bild (Abb. 2 (4)).

(3) Die physiologische Wirksamkeit des ultravioletten Lichtes und des Fluoreszenzlichtes im Auge.

Entsprechend dem objektiven ultravioletten Bild gibt es auch ein *subjektives* ultraviolettes Bild auf unserer Netzhaut. Es ist von satter, blauvioletter Farbe. Bei stärkerem Lichte wird es hellblau, bei sehr geringer Lichtstärke blaugrau oder grau.

Ausser dem farbigen Bildeindruck haben wir in starkem Ultraviolett noch eine auffällige weitere Erscheinung, die wir als *Lichtnebel* bezeichnet haben. Sie hängt mit der Fluoreszenz der Linse und der Cornea zusammen. Dass die Augenlinse in ultraviolettem Lichte ($\lambda = 366 \text{ m}\mu$ – $334 \text{ m}\mu$) sehr stark leuchtet, geht aus der Abb. 2 (1) hervor. Physiologisch hat das Auftreten eines leuchtenden Körpers in dem Auge selbst eine sehr seltsame Wirkung. Sobald das ultraviolette Licht der genannten Wellenlänge unser Auge trifft, so hat man eine höchst unangenehme Empfindung. Man glaubt einen leichten Nebel oder Schleier vor den Augen zu haben, der alles einhüllt und den ganzen sichtbaren Raum zu erfüllen scheint. Dieser Lichtnebel ist von ähnlicher Farbe wie das Fluoreszenzlicht der Linse, wodurch sich die Herkunft des Lichtnebels erweist. Man braucht nur eine andersfarbige Fluoreszenz dicht vor dem Auge zu erzeugen und der Lichtnebel nimmt die Farbe dieser Fluoreszenz an. Mit Hilfe einer gelbfluoreszierenden Uvonglasplatte gelingt dieser Versuch leicht. Der Lichtnebel ist dann gelblich.

Die genannten Erscheinungen haben wir im eigenen Auge nur im abgedunkelten Raume beobachtet. Bei Tageslicht oder dem vollen Lichte der Quarzlampe sieht man weder etwas von ultraviolettem Licht noch irgend eine Fluoreszenz. Beide Erscheinungen sind dann übertönt.

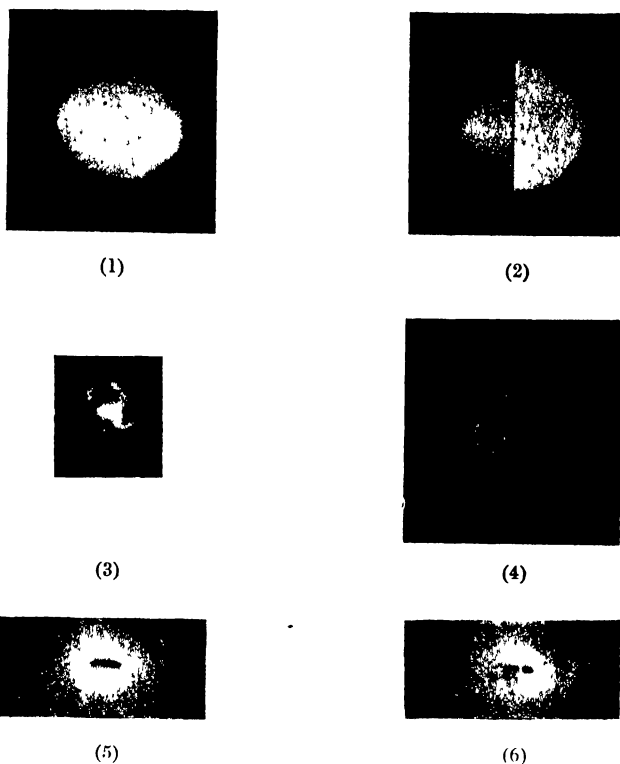


Abb. 2. Die im Ultraviolett fluoreszierende, helle sichtbare Strahlen aussendende Linse aus dem Auge des Schweines ist im eigenen Licht photographiert (1). (Aus Merker, 1928.) Ausser dem eigenen Licht lässt sie auch noch ultraviolettes Licht durch sich hindurch. Bild (2) zeigt den ultravioletten Anteil und den Anteil des sichtbaren Lichtes, das durch die Linse hindurchgeht oder von ihr ausgeht, nebeneinander. Links ist ein Ultraviolett-Fanger eingeschaltet. Hornhaut und Linse einer weissen Ratte entwerfen auch im Ultraviolett auf dem Augenhintergrund ein Bildchen des Brenners der Quarzlampe. Wenn die unpigmentierte Aderhaut und harte Augenhaut vorhanden sind, entsteht durch das Ultraviolett ein Fluoreszenzbild (3), wenn man sie abträgt, kann man auf der Netzhaut auch das Ultraviolett-Bild zeigen (4). Die Linse aus dem Auge einer Katze entwirft auf der Platte eines für diese Linse gebauten photographischen Apparates das ultraviolette Bild des Brenners der Quarzlampe. Im 6. Bild Schatten eines Holzstabes durch Überstrahlung hell, statt dunkel. Die Bildchen sind solarisiert (4-6).

Mit Hilfe des Lichtnebels ist es aber doch möglich, das Ultraviolett in einfacher Weise mit sichtbarem Lichte in Wettbewerb treten zu lassen. Wir sehen uns zu diesem Zweck die brennende Quarzlampe hinter einem Uvetfilter an. Die Leucht-röhre erscheint blauviolett. In passender Entfernung von der Lampe entsteht um

das Bild von der Leuchtröhre auch der Lichtnebel. Nähern wir uns der Lichtquelle, so erfolgt Verstärkung der beiden Lichteindrücke. Der Lichtnebel wird aber rascher stärker als das Ultraviolett. Als Folge davon geht das ultraviolette Bild im Lichtnebel unter.

Verdunkelt man in diesem Zustand und in diesem Abstand von der Lichtquelle das einstrahlende Licht durch ein Filter oder schliesst man die Augen für einen Augenblick, so erscheint nach Herstellung der alten Lichtstärke das Bild der Leuchtröhre sehr kräftig wieder. Im nächsten Augenblick ist es aber erneut im Lichtnebel verschwunden.

Tab. II erläutert dieses Versuchsergebnis noch näher. Sie bestätigt, dass bei älteren Menschen die Fluoreszenz der Linse rascher als beim Annähern an die ultraviolette Lichtquelle anwächst als bei jüngeren. Weiter ergibt es sich, dass die Fluoreszenzhelligkeit in jüngeren Jahren nicht zur Unterdrückung des Ultraviolett ausreicht. Die Durchlässigkeit für Ultraviolett ist höher.

Tabelle II. Die Zunahme der Linsenfluoreszenz und die Abnahme der Durchlässigkeit der Augenteile des Menschen für ultraviolettes Licht mit steigendem Alter.

10 verschiedene Versuchspersonen, männlich. Alter in Jahren	Das ultraviolette Bild der Leuchtröhre verschwindet beim Annähern an die Lampe	Ihr Abstand von der Lampe in cm.	Die Breite des Bildes der Leuchtröhre	Der Lichtnebel durch die Linse wird beim Annähern
20	Nicht	7	Bleibt	Verstärkt
21	"	7	"	"
21	"	7	Wird schmaler	"
23	"	7	Bleibt	"
25	"	7	Wird schmaler	"
38	Völlig	7	Wird vorher schmaler	Sehr stark
43	"	7	"	"
47	"	18	"	"
47	"	25	"	"
65	"	27	"	"

Noch in anderer Hinsicht ist dieses Versuchsergebnis sehr aufschlussreich. Es beweist, dass das ultraviolette Bild noch da ist, auch wenn es nicht mehr gesehen wird. Seine Reizstärke ist anscheinend zu schwach gegenüber der des sichtbaren Lichtes. Weiter zeigt sich, dass der Empfindlichkeitsverbrauch in der Netzhaut im ultraviolettem Licht viel grösser ist als im gewöhnlichen Lichte. Vielleicht hängt damit die Reizschwäche des ultravioletten Lichtes zusammen, die in auffallendem Gegensatz zu der photochemischen Wirksamkeit steht. Das verstärkte Erscheinen des ultravioletten Bildes nach kurzem Erholen der Netzhaut bestätigt das Gesagte. Das Ultraviolett ist danach zwar ein dunkles, aber nicht in jeder Hinsicht wirkungsloses Licht. Das Bildsehen ist, wie wir gezeigt haben, im langwelligen Ultraviolett durchaus möglich. Es tritt aber bei Tageslicht nicht in Erscheinung, weil die Empfindlichkeit unserer Augen für dieses Licht zu gering ist, und weil die chromatische Abweichung für diese kurzen Lichtwellen sehr stark ist und nicht ausgeglichen werden kann.

(4) *Die Schädigung des Wirbeltierauges durch Ultraviolett.*

Für das Auge der Wirbeltiere, wie auch für das der Wirbellosen, sind die langwelligen Abschnitte und die kurzwelligen Bezirke, die sich an das sichtbare Spektrum anfügen, gefährlich. Ultrarot ruft infolge der konzentrierenden Wirkung der Linse schon in kurzer Zeit der Einwirkung eine hochgradige Verletzung an der Netzhaut und an der Aderhaut hervor (Vogts Schüler, Trümper (1925) und Bücklers (1928)). Auch Ultraviolett erzeugt, wie bekannt, unangenehme Erscheinungen am Auge. Zweifellos sind die Lichtwellen des Dorno-Gebietes ($\lambda = 310-290 \text{ m}\mu$) am gefährlichsten. Doch auch langwelliges Ultraviolett ist nicht gleichgültig, wie sich an Planarien zeigen lässt (Merker und Gilbert, 1932). Die Schädigungen an Säugeraugen durch Ultraviolett-Strahlen hat zuerst Widmark (1891) studiert, während Birch-Hirschfeld (1904) sie im Wesentlichen aufgeklärt hat. Die Absorptionsverhältnisse der Hornhaut und der Linse bedingen es, dass vom kurzwelligen Licht hauptsächlich im vorderen Augenabschnitt Beschädigungen verursacht werden. Die tieferliegenden Teile des Auges sind verhältnismässig geschützt, weil die Linse im Mittel bei $\lambda = 322 \text{ m}\mu$ ihre Absorptionsgrenze im ultravioletten Licht hat. Kürzerwelliges Licht kann also die Netzhaut nicht erreichen. Trotzdem ist ultraviolettes Licht imstande, eine zerstörende Wirkung auf der Netzhaut zu entfalten. Studien mit spektralreinem Ultraviolett haben dies zweifelsfrei gezeigt. Nach Birch-Hirschfeld werden vor allem die grossen Ganglienzellen der Netzhaut angegriffen. Sie schwellen an, ihre Nisslschollen zerfallen, und es bilden sich Vakuolen im Protoplasma. Von englischer Seite (Duke Elder, 1926) sind diese Befunde bestätigt worden.

Die Schädigungen, die das Ultraviolett in den vorderen Augenteilen hervorruft, sind recht gut bekannt. Wir wissen darüber, dass sie erst einige Stunden nach der Bestrahlung sichtbar werden, dass auf der Bindehaut Rötung, Oedem und eiterige Absonderung, auf der Hornhaut Stichelung, Trübung und Geschwüre verschiedene Grade in der Erkrankung darstellen. Wegen der histologischen Einzelheiten sei auf die Arbeiten von Birch-Hirschfeld (1909), Politzer und Alberti (1924) und Hoffmann (1932) verwiesen. Dem letztgenannten Autor sind wir in der Darstellung gefolgt. Er hat lichtbiologisch weitere Aufklärung gebracht. Birch-Hirschfeld hatte bereits durch fortgesetzte Ultraviolett-Bestrahlungen, deren jede eine schwache Entzündung hervorrief, eine Veränderung an der Bindehaut des Kaninchens beobachtet, die mit dem Frühjahrskatarrh am Menschenauge grosse Ähnlichkeit hatte. Es zeigten sich grosse zapfenförmige Wucherungen am Epithel, hyaline Umwandlung der subepithelialen Gewebe, hyaline Degeneration der Gefässwandung und später eine ausgedehnte Pigmentierung. Hoffmann (1932) fand dazu, dass die Bestrahlungsfolgen alsbald einsetzen und nicht erst nach der Bestrahlung. Man kann sie mit Spaltlampe und Mikroskop sehr früh erkennen, sodass von einer Latenzzeit nicht eigentlich geredet werden darf. Als Massstab für die Reizstärke des verwendeten Lichtes konnte die Heilungsdauer der entstandenen Verletzungen dienen. Bei verschiedener Intensität erhielt er gleiche Wirkungen, wenn die Bestrahlungszeit um den Wert des Intensitätsunterschiedes multipliziert mit der 1.08 Potenz vergrössert wird. Zwischen Reizung, Belichtungszeit und Intensität herrschen (es

handelt sich um Hornhautgewebe) somit ähnliche Beziehungen wie zwischen Schwärzung, Belichtungszeit und Intensität bei den photochemischen Vorgängen in der photographischen Platte.

III. DIE BEWEGUNGEN VON PIGMENT UND VON STÄBCHEN UND ZAPFEN IN DER NETZHAUT DES FISCHAUGES UNTER DEM EINFLUSS VON ULTRAVIOLETTEM LICHT.

Der subjektive Nachweis über die Sichtbarkeit des ultravioletten Lichtes kann natürlich nur beim Menschen geführt werden. Bei allen anderen Lebewesen sind objektive Beweise nötig. Das objektive Beweisverfahren ist aber umständlicher und schwieriger, weil durch das Auftreten der Fluoreszenz in den farblosen Geweben des Tierleibes z.B. in der Netzhaut stets ein Mischlicht entstehen kann. Zwar unterdrückt der Sehpurpur die Fluoreszenz sehr erheblich, aber eben nur dort, wo er sitzt. Diese Verhältnisse sind noch ungeklärt. Zu dem möglichen Fluoreszenzlicht in der Retina, als in den lichtempfindlichen Zellen selbst oder in ihrer nächsten Nähe, kommt, völlig unabhängig von jeglichem Sehpurpur, noch der Lichtnebel, das Fluoreszenzlicht von Linse und Cornea.

Alle diese Fluoreszenzerscheinungen müssen auf ihre physiologische Wirksamkeit geprüft werden, wenn man der Frage über die Sichtbarkeit des Ultraviolett nachgehen will.

Uns schien das Studium der retinomotorischen Erscheinungen im Auge niederer Wirbeltiere unter dem Einfluss von ultraviolettem Licht gewisse Anhaltspunkte über die Wirksamkeit des Ultraviolett neben dem Fluoreszenzlicht zu bieten, das es erzeugt. Wir haben deshalb diese Bewegungen an Stichlingen geprüft und wollen diese wirklich vorzeigbaren Ergebnisse nun entwickeln. Dabei verweisen wir auf die beigegeführten Photographien (Abb. 3).

Es ist ja bekannt, dass sich das Wirbeltierauge als eine Blase vom Vorderhirn aus anlegt. Sie formt sich in der weiteren Entwicklung zum doppelwandigen Becher wie etwa ein Dewarsches Gefäß. Die Innenwand wird zur Netzhaut, während die Aussenwand sich in ein Pigmentepithel umwandelt, dessen Zellen Fortsätze zwischen die Sehzellen der Netzhaut schieben. Dadurch wird es ihnen möglich die zugewendeten Netzhautzellen, gerade die lichtempfindlichen Stäbchen und Zapfen zu umhüllen und nach Bedarf vor dem eindringenden Licht zu schützen.

So kann man in einem Auge, das dem Tageslicht ausgesetzt war, feststellen, dass die Stäbchen und Zapfen der Retina fast völlig im Pigmentmantel verschwunden sind und dass das Pigment selbst bis zur äusseren Grenzschicht vorgedrungen ist. Bei weniger starkem Licht entlässt das Pigment die lichtempfindlichen Stäbchen und Zapfen mehr und mehr aus seinem Schutze. In der Dämmerung zieht sich das Pigment sehr stark zurück und gibt die Sehzellen ganz frei.

Neben dieser Pigmentbewegung geht auch noch eine Bewegung der Stäbchen und Zapfen einher. Nach der Duplizitätstheorie vermitteln die Zapfen das Farbsehen am hellen Tage. Die Stäbchen sind lichtempfindlicher. Mit ihrer Hilfe sieht man noch in der Dämmerung. Aber sie gestatten es nicht Farben zu unterscheiden. Wir sehen dann nur noch hell und dunkel.

Auf entsprechenden Schnitten durch die Retina findet man nun bei manchen Wirbeltieren, dass im Tageslicht die Zapfen der äusseren Grenzmembran aufsitzen, während die Stäbchen im Dunkel des Pigmentes verschwunden sind. Bei Schnitten dagegen, die von Augen stammen, die im Dämmerlicht gehalten waren, erscheinen die Stäbchen auf der Grenzmembran, die Zapfen sind gestreckt. Wie man annimmt, befinden sich die Stäbchen und Zapfen, die auf der Grenzmembran aufsitzen am Orte des deutlichsten Sehens. Die Aussenglieder der Sehzellen, die wie auf dünnen oft fadenförmigen Hälsen von der Grenzmembran abgerückt sind, befinden sich ausserhalb der Zone des deutlichen Sehens.

Wir haben, wie erwähnt, an Stichlingen ähnliche Versuche wie die eben ange-deuteten auch mit reinem ultraviolettem Filterlicht gemacht. Das Ergebnis war sehr bezeichnend. Obwohl das Ultraviolett ein sehr dunkles Licht ist und man sich erst daran gewöhnen muss, ehe man in dem Raum mit ultraviolettem Licht etwas erkennt, zeigen sich die Zapfen vorn auf der Grenzmembran, geradeso als wenn starkes Licht geherrscht hätte. Zugleich ist auch das Pigment weit vorgezogen, als ob ein Lichtschutz nötig gewesen wäre. Die Stellung der Zapfen an der Grenzmembran entspricht durchaus unserer Farbempfindung in diesem Licht, nicht aber seiner Eigenschaft eines dunklen Lichtes.

Da nun, wie wir bereits wissen, die Fluoreszenz in den Wirbeltieraugen recht stark werden kann, da sie als Lichtnebel häufig in Erscheinung tritt, und da ferner sogar in den Lichtzellen selbst Fluoreszenz entstehen kann, so ist auch damit zu rechnen, dass die im Ultraviolett aufgetretenen Erscheinungen auch auf das Fluoreszenzlicht allein zurückgehen könnten. Jedenfalls sind diese Verhältnisse durch unsere bisherigen einfachen Versuche in ultraviolettem Licht, wie auch die Versuche der anderen Autoren nicht eindeutig geklärt¹.

Wir haben deshalb auch die Wirksamkeit des Fluoreszenzlichtes geprüft. Die Abb. 3 lässt erkennen, wie wichtig dieser Versuch in unserem Zusammenhange hier war. Sie zeigt dass das Fluoreszenzlicht (es war Chininfluoreszenz) fast die gleiche retinomotorische Kraft besitzt wie das reine Ultraviolett. Man kann also danach gar nichts darüber entscheiden, ob Ultraviolett als solches in den Stichlingsaugen überhaupt wirksam ist. Alle bisherigen Angaben dass die Fische ultraviolettes Licht sähen, sind also nicht mehr sicher.

Wir suchten auf Grund folgender Überlegung eine Entscheidung. Es wäre uns höchst seltsam gewesen, wenn die beiden Lichter: Fluoreszenzlicht und ultraviolettes Licht in jeglichem Abstände von der Lichtquelle die gleiche Wirkung behielten. Wir vermuteten vielmehr, dass in einem bestimmten Abstände von der Lichtquelle die Wirkungen deutlich auseinandergingen und dass sie dann eine Unterscheidung zuließen. Da wir diesen Abstand nicht kannten, mussten wir ihn herausfinden. Wir tasteten also schrittweise die schwächer werdenden Wirkungen des Lichtes in den Stichlingsaugen ab.

¹ So fand E. Hertel (1911, *Ber. Ophthal. Ges. Heidelberg*), dass an isolierten Netzhäuten von Fröschen und Fischen die Zapfenkontraktion noch auslösbar ist durch Licht von $226\text{ m}\mu$. Im Auge selbst war sie noch nachweisbar bei $396\text{ m}\mu$. Bei $330\text{ m}\mu$ hielt er eine indirekte Erregung durch Fluoreszenzlicht für möglich.

Die Abb. 3 zeigt das Ergebnis. Bei unserer Versuchsanordnung war die Wirkung des Ultraviolett in 3 m. Entfernung heftiger als die des Fluoreszenzlichtes. Das Pigment wurde im ultravioletten Licht stärker nach vorn an die Grenzmembran gelockt als im Fluoreszenzlicht. Die Zapfen standen im abgeschwächten Ultraviolett immer noch alle vorn an der Grenzmembran, im abgeschwächten Fluoreszenzlicht begannen sie zu weichen. Damit ist die Überlegenheit des Ultraviolett in seiner retinomotorischen Leistung gegenüber dem Fluoreszenzlicht sichergestellt.

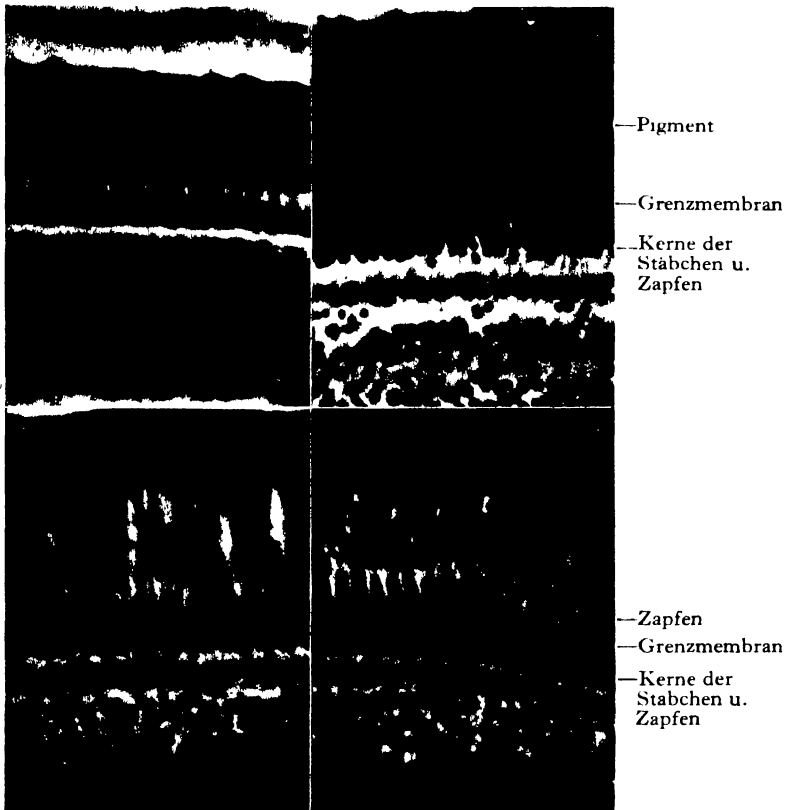


Abb. 3. Die retinomotorischen Erscheinungen in der Netzhaut von Stichlingen unter dem Einfluss von Fluoreszenzlicht und ultraviolettem Licht ($\lambda = 366 \text{ m}\mu$). (Aus: Merker, 1932.)

(1) Nach vorherigem Dunkelaufenthalt (2-5 St.) war der Stichling 2 St. in Chininfluoressenzlicht. Der Abstand von der Lichtquelle betrug 10 cm. Die Zapfen der Netzhaut sind deutlich sichtbar. Sie sitzen fest auf der Grenzmembran auf.

(2) Nach vorherigem Dunkelaufenthalt (2-3 St.) war der Stichling 2 St. in ultraviolettem Filterlicht ($\lambda = 366 \text{ m}\mu$). Der Abstand von der Lichtquelle betrug 25 cm. Das Pigment ist so weit vorgezogen, dass Stäbchen und Zapfen nicht sichtbar sind.

(3 und 4) Nach vorherigem Dunkelaufenthalt (2-3 St.) war der Stichling (3) in Fluoreszenzlicht, (4) in ultraviolettem Filterlicht ($\lambda = 366 \text{ m}\mu$). Abstand von 3 m. von der Lichtquelle.

Es zeigt sich, dass sowohl Pigment als auch Zapfen im Fluoreszenzlicht von der Grenzmembran abgerückt sind. Im Ultraviolett ist das Pigment der Grenzmembran nähergerückt und die Zapfen sitzen ihr fest auf.

IV. DIE PIGMENTBEWEGUNG IM SUPERPOSITIONSAUGE VON INSEKTEN UNTER DEM EINFLUSS VON ULTRAVIOLETTEM LICHT.

Auch bei den Insektenaugen glaubte man, dass das ultraviolette Licht nur durch seine fluoreszenzerregende Kraft wirksam sei. Das sichtbare Fluoreszenzlicht sollte das sichtbare Himmelslicht verstärken. Da insbesondere das Chitin vor den Augen farblos ist und wie wir festgestellt haben, deshalb stark fluoresziert, so glaubte man es finge alles Ultraviolett ab und verwandle es in sichtbares Fluoreszenzlicht.

Uns schien nach den Erfahrungen am eignen Auge diese Hypothese nicht gut begründet. Aber wie sollte man an einem Auge, das kaum grösser als ein Stecknadelkopf ist, Durchstrahlungen vornehmen, beobachten und vielleicht ausmessen können?

In dieser Schwierigkeit kamen uns die Physiologen zu Hilfe, die beobachtet hatten, dass es Insektenaugen gibt, die unter dem Einfluss von Licht einen Pigmentvorhang im Auge vorziehen können. Diese Einrichtung hat die unserer Irisblende entsprechende Aufgabe im Auge. Bei starkem Licht hält das Pigment den Lichtüberschuss von den Sehzellen ab. Wir suchten also mit ultraviolettem Licht eine solche Pigmentbewegung im Insektenauge in Gang zu bringen und hatten damit auch Erfolg. Bis zu $\lambda = 253 \text{ m}\mu$ kann es gelingen das Superpositionsauge in ein physiologisches Appositionsaug zu verwandeln.

(1) *Das Fluoreszenzlicht im Insektenauge.*

Wir haben nun nachgeforscht, wie der ebengeschilderte Blendenapparat im Auge der Insekten bei Bestrahlung mit ultraviolettem Licht wirkt. Dabei musste man feststellen, ob es gelingt wirklich ultraviolettes Licht auf dem Grund des Insektenauges nachzuweisen. Man musste aber auch unbedingt den Umfang und die Stärke des Fluoreszenzlichtes im Auge nachprüfen, um seine Rolle abschätzen zu können.

Es stellte sich nämlich heraus, dass das Fluoreszenzlicht nicht unterschätzt werden darf. Man kann leicht zeigen, dass nicht nur das Chitin vor den Augen fluoresziert, sondern dass alle Gewebe, sofern sie nicht gefärbt sind sehr stark fluoreszieren können. Meist sieht man ein bläulich-weisses Leuchten.

Die Tatsache, dass auch hier wie im Wirbeltierauge alles nicht gefärbte Gewebe fluoresziert, lässt erkennen, wie schwer die Frage zu entscheiden ist, ob wirklich das Ultraviolett gesehen wird. Von dem Wirbeltierauge konnten wir sagen dass das langwellige Ultraviolett gesehen wird, weil wir selbst eine besondere Empfindung von diesem Lichte hatten. Genau genommen können wir es also nur von uns sagen. Eine Biene wird uns über ihre Erlebnisse solcher Art keine Antwort geben. Auch die Dressurversuche, die man bei ihr angewendet hat, wären noch durchaus verständlich, wenn nur das in den Rhabdomen selbst entstehende Fluoreszenzlicht, das man sich stark genug vorstellen darf, gesehen würde. Und dass es gesehen wird, bedarf ja keines Beweises, weil es sichtbares Licht ist! Beweisen müssen wir nur, dass Ultraviolett wirklich bis zu den Sehzellen dringt. Wenn das so wäre, so gäbe es heute keinen Beweis dafür, dass die Bienen ultraviolettes Licht

sehen. Er ist nur deshalb möglich, weil, wie wir sehen werden, die Ultraviolett-Empfindlichkeit so ausserordentlich hoch ist, sodass man sicher ist, dass eine solche Wirkung vom Fluoreszenzlicht nicht erreicht werden kann.

- (2) *Ultraviolettes Licht wird trotz der Chitinfluoreszenz und der Fluoreszenz der Gewebe auf dem Grunde der Netzaugen von Insekten nachgewiesen.*

Auch unsere Versuche über die Pigmentbewegung in den Insektenaugen durch ultraviolettes Licht sind keineswegs beweisend für das Gesehenwerden des ultravioletten Lichtes. Wir können aber durch sie zeigen, dass tatsächlich das Ultraviolett bis auf den Grund der Augen dringt.

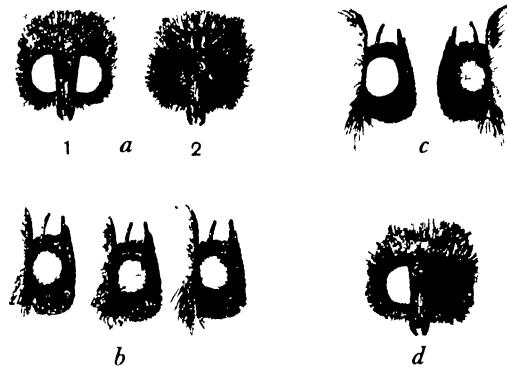


Abb. 4. Die Pigmentbewegung im Auge von Dämmerungsschmetterlingen und ihre Auswirkung. (Aus: Merker, 1929 a.) Die hier dargestellten Schmetterlingsaugen beobachtet man am besten mit einem Augenspiegel, oder mit schwacher Vergrösserung eines Opakilluminators oder Ultropaks.

(a) (1) Die leuchtenden Augen eines an dem Fenster eines erhellten Zimmers auf und ab tanzenden Dämmerungsschmetterlings. (2) Die Augen des gleichen Tieres bei Tageslicht, oder nach einer kräftigen Bestrahlung mit Lampenlicht während etwa $\frac{1}{2}$ Stunde.

(b) Das schrittweise Kleinerwerden der leuchtenden Fläche im Auge bei Betrachtung mit kräftigem Licht, hier reinem Ultraviolett $\lambda = 366-334 \text{ m}\mu$.

(c) Die Unabhängigkeit der beiden Augen hinsichtlich ihrer Pigmentbewegung im Innern.

(d) Ein einseitig bestrahlter Schmetterling von vorn.

Um dies verständlich zu machen, erwähnen wir, dass uns dabei noch ein Umstand im Insektenauge günstig war. Manche dieser Augen, so die Augen von Eulenschmetterlingen, sind so ausgerüstet, dass das eingedrungenen Licht noch ein zweites Mal durch die Rhabdome geschickt wird. Auch diese Einrichtung ist als eine Ausnützung schwachen Lichtes zu verstehen. Im Augengrund ist eine spiegelnde Schicht, die eingedrungenes Licht wieder zum Auge hinausstrahlt. Darum sehen die Augen von an den Fenstern erleuchteter Zimmer tanzenden Schmetterlingen wie feurige Kohlen aus.

Die spiegelnde Schicht in den Augen ist besonders beschaffen. Sie ist eine Art Samt, bei dem aber die steilstehenden Härchen hohl und lufthaltig sind. Sie stellt also eine Art Tracheensamt dar.

Wir vermuteten, dass gerade diese Schicht auch im ultravioletten Licht ganz besonders stark leuchte, und haben uns nicht getäuscht. Die beigegebenen Bilder (Abb. 4) mögen dies zeigen. Das physiologische Dunkelaugen dieser Schmetterlinge

zeigt bei Bestrahlung mit ultravioletterm Licht in der Mitte eine graue Scheibe, die im sichtbaren Licht feuerrot aussieht. Diese Scheibe wird kleiner und kleiner wenn man länger belichtet, genau wie auch im sichtbaren Licht. Da wir nur reines Ultraviolett ins Auge gesandt haben, so kann das Leuchten nur Fluoreszenzlicht sein. Dass dieses Fluoreszenzlicht verschwindet hängt mit dem Zurückwandern des Pigmentes zusammen. Sobald die Pigmentstrümpfe weit genug zurückgezogen sind, kommt kein Licht mehr aus dem Auge heraus. Man sieht höchstens in den gerade durchstrahlten Keilen ein punktförmiges Aufleuchten, das aber nur durch das Mikroskop wahrzunehmen ist. Die einheitliche graue Scheibe ist verschwunden; das Auge ist dunkel. Auch in gewöhnlichem Licht verläuft der Vorgang, wie erwähnt, nicht anders.

Ist diese graue Scheibe nun tatsächlich durch die Fluoreszenz der Rückstrahlerschicht entstanden? Denn eigentlich zurückgestrahltes Licht kann ja es nicht sein. Somit müssen wir den Entstehungsort dieser Fluoreszenz auszumachen suchen.

Da das Licht der grauen Scheibe im Auge selbst entsteht und doch durch eine, wie wir schon dargetan haben, Sieblende betrachtet werden muss, so muss das Licht auch verhältnismässig stärker sein als das Licht der Gewebe.

Mit folgendem Versuch wollen wir die Möglichkeit erweisen, dass die Tracheenschichte stärker leuchtet als die Gewebe. Wir halten Glaswolle in ultraviolettes Licht. Man sieht ein starkes Leuchten. Jedenfall ist es stärker als das Leuchten von gewöhnlichem Glas. Wir vermuten, dass durch die vielen totalen Reflexionen, die die Lichtstrahlen in der Glaswolle erleiden (es ist ja ein sehr oft wechselndes System von Luft und Glas), die Fluoreszenz erhöht wird. Wir beweisen diese Deutung durch folgenden weiteren Versuch. Lässt man in die Glaswolle eine Substanz von etwa gleicher Brechzahl wie Glas sie besitzt einsaugen, treibt dadurch die Luft zwischen den Glasfäden aus, so nimmt die Fluoreszenz sofort ab, vorausgesetzt, dass die hinzugefügte Flüssigkeit nicht selbst fluoresziert.

Wenn damit nun auch die Stärke der Leuchtkraft der obengenannten grauen Scheibe wahrscheinlich gemacht, so war doch noch nicht erwiesen, dass die Rückstrahlerschicht tatsächlich die graue Scheibe erzeugt. Dies können wir aber am lebenden oder überlebenden Schmetterlingskopf nachweisen. Wir brauchen nur das Tracheentapetum rasch mit einer Flüssigkeit von ähnlicher Brechzahl zu durchtränken wie sie das Gewebe hat. Das gelingt mit einem raschwirkenden Fixierer von genannter optischer Eigenschaft. Durch rasches Abtöten erlaubt er nicht mehr, dass das Pigment noch einmal seinen Ort wechselt. Trotzdem sieht man die leuchtende Scheibe verschwinden. Sie verschwindet aber auf andere Weise, wie wenn sie durch das Pigment verdeckt wird. Sie wird in diesem Fall in ihrer ganzen Ausdehnung blasser und blasser, um schliesslich unsichtbar zu werden. Beim Zurückschieben des Pigmentes dagegen wird sie von der Seite her eingengt und auf diese Weise kleiner und kleiner. Dass tatsächlich nur mit einem optischen Aussertätigkeitssetzen der spiegelnden Schicht gerechnet werden darf, dass also nur eine Homogenisierung stattgefunden hat, erweist sich aus der Tatsache, dass nach Ersatz des Fixierers durch niedrigstufigen Alkohol das Tracheentapetum wieder aufleuchtet. Es war also nicht verdeckt sondern nur seiner optischen Wirk-

samkeit beraubt. Nun kann man das Spiel so oft wiederholen als man will. Man ersetzt den Alkohol durch beispielsweise Xylol, so verschwindet das Leuchten im Auge. Geht man aber vorsichtig wieder in den Alkohol zurück, so leuchtet es erneut auf und so fort.

Durch diese Versuche ist bewiesen, dass ultraviolettes Licht tatsächlich in nennenswerter Menge bis auf den Grund des Insektenauges dringt und dort erhebliche Fluoreszenz hervorzurufen vermag. Die Reflektorenschicht im Insektenauge fluoresziert besonders stark.

Die Geschwindigkeit der Pigmentwanderung im lebenden Auge von Dämmerungsschmetterlingen ist verschieden gross. Wir lassen eine Aufstellung über die Zeiten folgen, die wir an Eulenschmetterlingen festgestellt haben. Schwärmer lassen viel geringere Zeiten erkennen.

Das leuchtende Auge einer grossen Zahl von Dämmerungsschmetterlingen wird dunkel bei Bestrahlung mit:

Sichtbarem Licht ($\lambda = 800 \text{ m}\mu$ und $400 \text{ m}\mu$) in 3-4 Minuten.

Reinem ultraviolettem Filterlicht ($\lambda = 366 \text{ m}\mu$) in 3-14 Minuten.

Auch im reinen spektralen Ultraviolett haben wir die Pigmentwanderungen einwandfrei beobachtet und zwar bis zum Licht der Wellenlänge $\lambda = 253 \text{ m}\mu$. Die hier folgenden Zeitangaben (Tab. III) gelten für den Eulenschmetterling *Agrotis plecta*.

Tabelle III. Die Geschwindigkeit der Pigmentwanderung im Auge von *Agrotis plecta* (8 Tiere) in ultraviolettem Licht.

Das Augenleuchten verschwindet im Ultraviolett bei $\lambda =$				
366 m μ	313 m μ	265 m μ	253 m μ	228 m μ
Nach 13 Min	Nach 14 Min Nach 18 Min. Nach 20 Min	Nach 50 Min noch $\frac{1}{2}$ hell	Nach 20 Min. Nach 35 Min.	Nach 60 Min. $\frac{1}{2}$ hell Nach 120 Min. $\frac{1}{2}$ hell

Man sieht, dass die Werte um $\lambda = 228 \text{ m}\mu$ schwankend sind. Nicht in allen Fällen ist überhaupt ein Erfolg verbürgt. Er hängt ab von der Grösse und Tiefe der Augen.

V. DIE AUSDEHNUNG DES SICHTBAREN SPEKTRUMS FÜR *DROSOPHILA* UND DIE HONIGBIENE SOWIE DIE VERTEILUNG DER BEWEGUNGS-ANREGENDEN KRAFT IN DIESEM SPEKTRUM.

Nach den Untersuchungen von Bertholf (1931, 1932) ist ultraviolettes Licht für die Bienen, wie auch für die Taufliege *Drosophila* sichtbar. Für beide Insekten ist das langwellige Ultraviolett von sehr grosser Wirkung. Sie überragt die aller anderen sichtbaren Bezirke. Doch zeigt die Taufliege *Drosophila* etwas andere Empfindlichkeiten in den verschiedenen Abschnitten des Lichtspektrums als die Biene oder gar der Mensch. Am kurzwelligen Ende reicht das Spektrum der *Drosophila* erheblich weiter als das der Biene. Vermutlich hängt dies mit der

Kleinheit der Augen von *Drosophila* zusammen, der Art ihrer Kristallkegel und der Dicke des Chitins vor den Augen. In der Kurve der bewegungsanregenden Kraft im Spektrum werden für *Drosophila* 3 Maxima festgestellt. Das Hauptmaximum erreicht, wie bei der Biene, erstaunlich hohe Werte. Es ist 4-mal so hoch als das im Blaugrün. Das Maximum im sichtbaren Gebiet ist gegenüber dem der Biene und unserer eigenen Augen ($\lambda = 550 \text{ m}\mu$) beträchtlich ins kurzwellige Gebiet verschoben. Die Biene hat nur zwei Maxima, der Mensch nur eines, im Gelbgrün.

Wie schon erwähnt, liegt das Hauptmaximum bei der Biene im Ultraviolett. Es ist 3-mal so hoch als das Nebenmaximum im sichtbaren Gebiet, das mit dem Maximum des Menschen bei $\lambda = 550 \text{ m}\mu$ liegt. Das Hauptmaximum für *Drosophila* ist noch höher als bei der Biene. Das 3. Maximum für *Drosophila* liegt jenseits des Ultravioletts unseres Tageslichts im kurzwelligen Gebiet. Es hat für die Tiere biologisch keine Bedeutung, da die Fliegen nicht in einer Umwelt mit solchem Licht leben. Damit ist für ein zweites Tier gezeigt, dass es Lichtempfindlichkeiten besitzt, die es nie geübt hat und die es für gewöhnlich nicht verwerten kann. Bechers *Daphnia pulex* reagierte noch etwas weiter im kurzwelligen Ultraviolett als die Taufliege.

Die obengenannten Versuche liefen darauf hinaus, festzustellen, welcher weissen Vergleichshelligkeit die bewegungsanregende Wirkung die verschiedenen Ultraviolett-Linien im Quarzlampenspektrum gleichkommen. Da aber die Energie der Ultraviolett-Linien natürlicherweise nicht gleich gross ist, und die experimentelle Gleichmachung nicht angestrebt wurde, so musste man mit Hilfe einer Ausgleichsrechnung zum Ziel kommen. Dazu war die Festlegung des anregenden Effektes der Ultraviolett-Linien, gemessen in Weissheit und die Ausmessung der Energie der Ultraviolett-Linien in Prozenten der stärksten Ultraviolett-Linie $\lambda = 366 \text{ m}\mu$ nötig. Zur Messung des biologischen Teiles der Aufgabe wurde die Versuchsanordnung von Mast (1917), jedoch mit der Einrichtung für ultraviolettes Licht benutzt. Den Tieren wurde in einem Kasten mit matten Fenstern die Vergleichslichter weiss und Ultraviolett geboten. Nach Versuchsende schob man Wände derart in den Kasten ein, dass die zwei Ansammlungen von Insekten an den Fenstern in je einem Kämmerchen gefangen sassen und ausgezählt werden konnten. Es herrschte auf beiden Fenstern Energiegleichheit (Helligkeitsgleichheit?) für die Tiere, wenn die eine Hälfte der Insekten an einem, die andere Hälfte am anderen Fenster sass. Nach Umdrehen des Kastens wiederholte sich das Spiel. Die Tiere wanderten erneut zum Lichte, weil auch an der gegenüberliegenden, jetzt erhellten Wand zwei weitere matte Fenster leuchteten. Zur Lösung der physikalischen Aufgabe war die Ausmessung der Energien der Spektrallinien mittels Thermoelement und Galvanometer nötig.

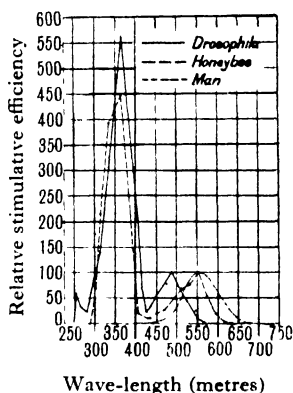


Abb. 5. Die Verteilung der bewegungsanregenden Kraft im Spektrum für *Drosophila*, Honigbiene und das Helligkeitsmaximum für den Menschen. (Aus Bertholf, 1932.)

Die Quotienten dieser beiden Wertereihen ergaben den relativen anregenden Effekt, bezogen auf die Einheit der Energie. Ihre Werte ergaben im Koordinatensystem die beigefügten Kurven (Abb. 5). Jedoch sind die Werte nicht ganz reine Ultraviolett-Werte. Es birgt sich in ihnen stets noch die Wirkung der Augenfluoreszenz, die nicht ausgeschaltet werden kann. Wenn sie auch die hohen Ultraviolett-Werte nicht erheblich beeinflusst, so ist es auch nicht richtig, sie gänzlich zu übergehen. Die Tatsache, dass beide Insektenarten neben den Netzaugen Ocellen besitzen erweckt neue bisher unbeantwortete Fragen. Aber trotzdem darf man wohl im Hinblick auf die Versuche von Kühn (1927), die in vieler Hinsicht durch vorliegende Ergebnisse erst richtig verständlich werden, sagen, dass Bienen und 'Taufiegen das Ultraviolett sehen.

VI. DIE DAPHNIEN SEHEN ULTRAVIOLETTES LICHT.

Man kann die Daphnien nicht dressieren. Man muss ihnen daher zum Nachweis ihrer Fähigkeiten im Sehen von ultravioletter Licht auf eine andere Weise beikommen. Auch bei ihnen spielt wie bei allen bisher untersuchten Tieren die Fluoreszenz der Gewebe eine gewisse Rolle. Das Tier erscheint in reinem Ultraviolett in einem mondscheinartigen Glanze und zwar leuchtet das Körpergewebe stärker als der dünne, farblose Chitinpanzer.

Die Augen der Daphnien sind sehr klein. Man kennt keine Pigmentbewegung darin. Wir können also in der Weise wie bei den Dämmerungsschmetterlingen die Frage nicht lösen, ob das Ultraviolett bis auf den Grund der Augen dringt oder nicht.

Diese rein physikalische Vorfrage war uns nicht unwichtig, wir haben deshalb den ganzen Körper der Daphnien durchstrahlt. Das Ergebnis war, dass das gesamte ultraviolette Spektrum fast vollständig durch ein Tier von der Dicke und der Dichte einer Daphnie hindurch geht; sie wird völlig durchschossen. Damit sind wir auch sicher, dass das Ultraviolett bis zu den lichtempfindlichen Sehzellen in den Augen dringt.

Um nun die Lichtsinnesempfindlichkeit im Ultraviolett und im Fluoreszenzlicht gegen einander auswerten zu können, mussten wir einen Zweilichterversuch einrichten. Dabei wurde den Tieren zugleich Ultraviolett und Fluoreszenzlicht geboten, wie es ja infolge der Gewebefluoreszenz in jedem reinen Ultraviolettversuch tatsächlich der Fall ist, wir mögen es wollen oder nicht.

Wie verrät uns aber die Daphnie, dass sie entweder das eine oder das andere der beiden Lichter vorzieht, die wir ihr von zwei Richtungen her darbieten? Wir nützen dazu eine seltsame Eigentümlichkeit der Daphnien aus. Die Daphnien sind sehr stark vom Lichte abhängig, sie sind geradezu wie an die Lichtstrahlen angeheftet. Lässt man Licht von rechts oder von links auf sie einfallen, so drehen sie stets den Rücken diesem Lichte zu und hüpfen im Wasser auf und ab. Kommt Licht streng von oben, so neigen sie den Körper, dass er den Rücken den Strahlen zuwendet. Bei Licht von unten finden wir sie deshalb auf dem Rücken schwimmend.

Lässt man Fluoreszenzlicht, das ja sichtbares Licht ist, von unten auf die Daphnien einstrahlen so schwimmen sie auf dem Rücken. Das ist natürlich. Aber

auch in reinem ultraviolettem Licht von unten, benehmen sie sich so. Danach konnten wir den Zweilichterversuch so einrichten, dass wir das Ultraviolett von unten, das Fluoreszenzlicht aber von oben boten, oder umgekehrt. Dadurch dass die Tiere sich dem stärkeren Licht mit dem Rücken zuwandten, konnten wir entscheiden, welches von beiden das wirksamere Licht war. Macht man das Licht von oben und unten gleich stark, so schwimmen die Tiere wie gewöhnlich auf dem Bauche, also mit dem Rücken nach oben. Das ist ihre natürliche Lage. Auch in der Dunkelheit schwimmen sie so. Es muss daher das Licht von unten schon einen grösseren Reiz haben, wenn sich die Daphnien auf den Rücken legen sollen.

Die Entscheidung fällt in dem Zweilichterversuch:

Ultraviolett kommt von unten	}	die Daphnien schwimmen auf dem Rücken.
Fluoreszenzlicht kommt von oben		

Ultraviolett kommt von oben		die Daphnien schwimmen auf dem Bauch.
Fluoreszenzlicht kommt von unten		

Fluoreszenzlicht kommt von oben (stärker)		die Daphnien schwimmen auf dem Bauch.
Fluoreszenzlicht kommt von unten		

In diesen Versuchsergebnissen liegt der 1. Beweis, dass die Daphnien das ultraviolette Licht sehen. Denn wenn die Daphnien das ultraviolette Licht nicht sähen so hätten sie nicht auf dem Rücken schwimmen dürfen, da ihnen von oben stärkeres Fluoreszenzlicht geboten wurde als in ihren Augen entstehen kann.

Es ist noch ein sehr klarer 2. Beweis möglich: Die Daphnien antworten auf eine Verminderung der Lichtstärken ganz allgemein mit Absinken; sie antworten aber auch bei Vergrösserung der Lichtstärke mit Absinken. Auch bei ultraviolettem Licht bedingt eine plötzliche Lichtverstärkung Absinken, wie wir von den eindringlichen Versuchen von Becher (1921) her wissen, und Wegnahme von Licht beantworteten die Daphnien ebenfalls Absinken.

Damit ist die Frage entschieden, ob das stark wirksame ultraviolette Licht die Hüpfbewegungen im ersten Augenblick schon lähmt, oder ob dieses Verhalten als gewöhnliche Lichtwirkung zu verstehen ist. Man kann nun nicht mehr von einer Lähmung reden, da auch Verminderung des Lichtes ein Absinken bewirkt. Je nach dem Lichtunterschied, der durch Zugeben und Wegnehmen des Lichtes entsteht, hält das Absinken länger oder weniger lang an. Bei geringerer Änderung sinken die Daphnien nur kurz und wenig ab, bei starkem Unterschied länger und tiefer. Dabei ist es einerlei ob das Licht von oben, oder von unten, oder gar von der Seite kommt.

Lassen wir bei Dunkelheit plötzlich Ultraviolett einfallen, so sinken die Tiere beträchtlich ab. Das Gleiche tritt ein, wenn Ultraviolett zu sichtbarem Licht hinzukommt (Becher, 1923). Lassen wir aber nach einer kurzen Gewöhnung im vollen Ultraviolett plötzlich Dunkelheit eintreten, so sinken die Tiere auch beträchtlich ab. Wir haben bei unseren Versuchen ein Absinken von 4 Sekunden gemessen. Dabei liessen wir das Ultraviolett stets von unten einfallen. Der gleiche Versuch mit Fluoreszenzlicht von unten liefert ein ganz anderes Ergebnis. Die Daphnien sinken in diesem Licht, wenn überhaupt, nur 1 Sekunde lang ab. Das

Fluoreszenzlicht das wir boten, war natürlich auch wieder stärker als das Fluoreszenzlicht in den Augen werden kann. Damit ist die Überlegenheit des Ultraviolett als Reiz dargetan (Abb. 6).

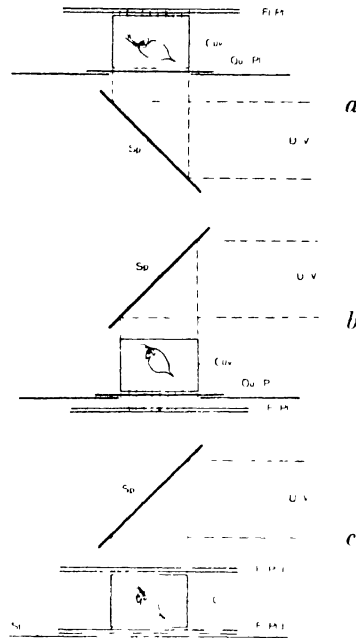


Abb. 6. *Daphnia pulex* im Zweilichtversuch (Aus: Merker, 1930.)

(a) *Daphnia pulex* wird von unten mit reinem ultraviolettem Licht ($\lambda = 366-334 \text{ m}\mu$) bestrahlt. Von oben kommt Fluoreszenzlicht einer Chimsulfatplatte. Eine Anzahl von Tieren stets auf dem Rücken.

(b) *Daphnia pulex* wird von oben mit reinem Ultraviolett ($\lambda = 366-334 \text{ m}\mu$) bestrahlt; Fluoreszenzlicht kommt von unten. Die Tiere schwimmen wie gewöhnlich mit dem Bauch nach unten.

(c) *Daphnia pulex* wird von oben mit stärkerem Fluoreszenzlicht und dementsprechend schwächerem Ultraviolett, von unten mit schwächerem Fluoreszenzlicht bestrahlt. Die Daphnien schwimmen mit dem Bauch nach unten. Um 180° gedreht, ist das Bild auch richtig.

Fl.Pl. = Fluoreszenzplatte; Cuv. = Cuvette, Qu.Pl. = Quarzplatte; Sp. = Spiegel; UV. = Ultraviolettes Licht.

VII. DAS SEHVERMÖGEN DER PLANARIEN IN ULTRAVIOLETTEM LICHT.

Bei der Prüfung des Lichtsinnes der Süsswasserplanarien im Ultraviolett sind besondere Umstände zu beachten. Aus früheren Erfahrungen (Merker und Gilbert, 1932) weiss man, dass diese feuchthäutigen Tiere im ultravioletten Licht rasch geschädigt werden und zerfallen. Man muss daher feststellen, ob das Versuchslicht in der kurzen Zeit eines Lichtsinnesversuchs Verletzungen hervorruft. Aber selbst wenn dies, wie bei unseren Beobachtungen, nicht der Fall wäre, so könnte das Licht doch schmerzhaft sein. In der Literatur hat man dergleichen vermutet. Infolge des Fluoreszenzlichtes, das in allen farblosen Geweben bei Ultraviolett-

bestrahlung entsteht, kann die Möglichkeit nicht abgewiesen werden, dass die Planarien im Ultraviolett eben dieses sichtbare Fluoreszenzlicht ihrer Lichtsinneszellen wahrnehmen, nicht aber das ultraviolette Licht selbst. Dieser Verdacht ist um so berechtigter, als die Lichtwege in den kleinen Planarienaugen sehr kurz und ultraviolett-absorbierende Organe wie Hornhaut, Linse und Glaskörper fehlen. Die Lichtstärke der Fluoreszenz kann daher verhältnismässig gross sein. Gewisse Versuche erweisen neben dem Augenlichtsinn auch einen Hautlichtsinn bei den Planarien, man muss also seinen Anteil am Gesamtlichteindruck klären.

Was verraten die Versuche? Wie im gewöhnlichen Licht (Tageslicht, Lampenlicht oder volles Quarzlicht) werden die Planarien auch vom reinen, langwelligen Ultraviolett (366–313 m μ) stark erregt und flüchten stracks vom Lichte fort ins Dunkle. Dabei dienen ihnen die Lichtstrahlen als Wegweiser in der Weise wie Taliaferro (1920) es für sichtbares Licht beschrieben hat. Bei dieser Flucht ist der Kopf vom Lichte abgewendet. So werden die Sehzellen vom Augenpigment, das die Sehzellen halbkugelig als Pigmentbecher umschliesst, im Schatten gehalten. Auf der Lichtflucht suchen die Tiere, so lange es irgend geht, mit ihren Lichtsinneszellen im Dunkeln zu bleiben. Sie stellen aber andauernd, durch Tasten des Kopfes nach rechts und links, die Lichtgrenze fest, die ihr Augenbecher erzeugt. Gutwillig wird sie nicht überschritten. Mit ihrer Hilfe gelangen die Tiere aber sicher gradlinig ins Dunkle. Das Kriechen der Planarien auf der Flucht vor dem Ultraviolett ist ruhiger, fördernder und nicht so überstürzt wie in vollem Quarzlicht, das 6-mal so hohe photochemische Lichtintensität aufwies als das Ultraviolett (0.5 Bunsen-Roscoe Einheiten pro Sekunde). Im Quarzlicht und im Ultraviolett entsteht bei genügender Stärke eine Lichtspur, die sich von der Kriechspur in schwachem Licht und von der Dunkelspur, die durch Fortkriechen im Dunkeln entsteht, unterscheidet. Diese Lichtspur ist flüchtiger und, da die Wendungen der Tiere nach den Seiten heftiger sind und der ganze Körper peristaltische Bewegungen macht, zerrissener. Im Dunkeln kriechen die Planarien ohne Richtung und unter Verlangsamung weiter.

Auch Fluoreszenzlicht (1–2 Lux), ähnlich der Gewebefluoreszenz, doch stärker als solche, hat als sichtbares, viel blau und violett enthaltendes Licht unverkennbare Lichtwirkungen auf die Planarien. Sie flüchten auch vor ihm. Doch zeigen Zweilichterversuche bei rechtwinkliger und gegenüberliegender Anordnung der Lampen, dass sich das Fluoreszenzlicht in seiner Wirkung nicht mit der Richtung des langwelligen Ultraviolett auf die Planarien messen kann. Genau die gleichen Erfahrungen haben wir auch bei Daphnien gemacht. Seitliches Fluoreszenzlicht lenkt die Planarien kaum aus ihrer Fluchtbahn. Ultraviolett dagegen trieb sie bis zu 45° hinaus, wenn es die Stärke des 1. Lichtes erreichte. In Zweilichterversuchen mit gegenüberstehenden Lichtquellen, wobei Glühlicht und Ultraviolett, zwei Glühlichter oder zwei Ultraviolett-Lichter verwendet wurden (Abb. 7, 2), wanderten die Planarien auf graden Bahnen senkrecht zur Lichtrichtung hin und her. Dabei sah das eine Auge nach der einen Lichtquelle, das andere nach der gegenüberstehenden Lampe. Doch bogen die Tiere erst dann in diese "Photometerstellung" ein, wenn sie den Punkt zwischen den Lichtern erreicht hatten, der von

beiden Seiten etwa gleiche Reizstärke erhielt. Ehe dieser Punkt erreicht war, liessen sich die Planarien dem schwächeren Licht entgegentreiben. Unter solchen Umständen hielt unser dunkles Ultraviolett einer Helligkeit von 200–300 Hefnerkerzen sichtbaren Lichtes in seiner Richtkraft und abstossenden Wirkung auf die Planarien das Gleichgewicht. Dabei betrug die Helligkeit dieses Ultraviolett,

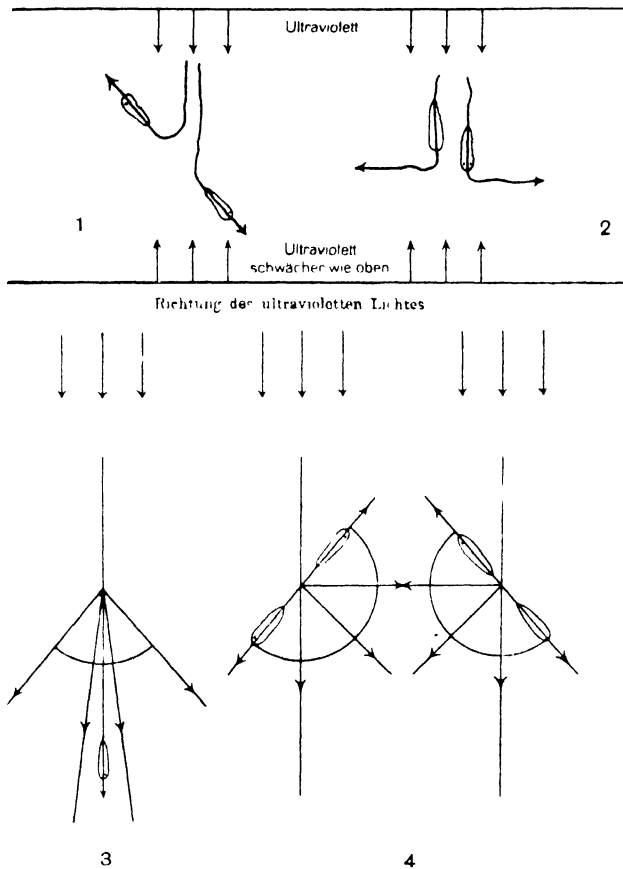


Abb. 7. Die Fluchtfelder einäugiger (4) und zweiaugiger Planarien (3) in seitlichem Ultraviolett und im Zwellichterversuch. (1 und 2.) Aus Merker und Gilbert, 1932.

gemessen als Fluoreszenzlicht auf Papier, noch nicht 1 Lux. Wurde Ultraviolett auf der einen, sichtbares Licht auf der andern Seite verwendet, so hielt sich der Ultraviolett-Gehalt beider Lichter im Verhältnis 200 : 1. Trotzdem das obengenannte Ergebnis! Es lenkt also nicht Schmerz und nicht Schädigung durch ultraviolettes Licht die Planarien in ihre Bahn. Auch Fluoreszenzlicht für sich allein ist zu schwach, um die mitgeteilten Versuchsergebnisse verständlich zu machen.

Es ist daher sicher, dass langwelliges Ultraviolett von den Planarien beachtet wird. Doch durch welche Organe dies geschieht, ist noch nicht klar. Hier halfen die

Ergebnisse von kopflosen Tieren und augenlosen Tieren mit unversehrttem Gehirn weiter. Bei beiden liess sich wiederum die Hell- und Dunkelspur unterscheiden. Auch sie empfanden das Verlöschen des Lichtes und verzeichneten diese Tatsache als Ruck auf ihrer Spur. Doch waren die Antworten in den Zweilichterversuchen schlecht und die Lichtflucht im Ultraviolett war längst nicht so sicher und bestimmt wie die der Tiere mit Augen. Erst als die Augen der blinden Tiere neu entwickelt waren, herrschte wieder die alte Sicherheit im Abwandern vor dem Lichte. Somit ist sicher, dass die Haut der Planarien auch ultraviolett-empfindlich ist. Die Hauptleistung beim Einstellen zum Licht fällt aber den Augen zu.

Weitere Beweise dafür lieferten die einäugigen Tiere. Sie benahmen sich im seitlich einfallenden Ultraviolett wie die unversehrten Planarien. Sie flüchteten in ganz derselben Weise ins Dunkle wie sie. Ihre Fluchtfelder (Abb. 7 (4)) jedoch lagen anders als die der zweiäugigen Planarien. Die rechtsäugigen Tiere benutzten ein Fluchtfeld nach links, die linksäugigen ein symmetrisch dazu gelegenes nach rechts. Das ist nur möglich, wenn die Augen zu einem sehr wesentlichen und übergeordneten Anteil am Lichteindruck beteiligt sind.

Im gerichteten Ultraviolett von oben schlugen die rechtsäugigen Planarien meist Linkskreise, die linksäugigen Rechtskreise bei ihren Bewegungen ein. Die Grösse dieser Kreise ist bei dem gleichen Tier abhängig von der Lichtstärke. Man erhält also kleinere Kreise bei starkem und grössere Kreise bei schwachem Licht. Sobald das Licht verlöschte, krochen die Planarien gradlinig weiter. Diese Ergebnisse stehen mit den obengenannten in bestem Einklang und belegen dasselbe wie sie.

Im rechtwinkelig angeordneten Zweilichterversuch antworteten die Planarien bei Belichtung der blinden Seite längst nicht so klar und bestimmt wie bei Bestrahlung der augentragenden Seite. Das gilt für Ultraviolett von 0.07 Bunsen-Roscoe Einheiten und Glühlicht von 250–350 Lux. Fluoreszenzlicht hatte keinen Einfluss.

Im Zweilichterversuch mit gegenüberliegenden Lichtquellen (Abb. 7 (1)) hatten die einäugigen Planarien nicht wie die unversehrten Tiere eine senkrecht zur Strahlenrichtung gelegene Bahn gewählt, die ihnen von beiden Seiten etwa gleiche Helligkeit gewährleistete, sondern sie krochen einen graden Weg, der die Lichtrichtung schief unter einem Winkel von 40° schnitt. Diese Kriechbahn gestattete dem einen noch vorhandenen Auge dauernd im Dunkeln zu bleiben. Sowohl von hinten als auch von vorn streift das Licht am Auge vorbei. Solcher Bahnen gibt es zwei. Und beide wurden gebraucht! Die linksäugigen Tiere benutzten die Linie, die von rechts nach links die Lichtstrahlen unter dem genannten Winkel schnitt, die rechtsäugigen zogen die Bahn, die von links nach rechts unter gleichem Winkel die Richtung der Lichtstrahlen überquerte.

Man erkennt daraus klar, dass die Lichteindrücke durch das Auge auch im Ultraviolett allen anderen übergeordnet sind. Die Planarien verhalten sich demnach bei raschen Antworten auf Belichtung so, als hätten sie keinen Hautlichtsinn. Sie richten sich nur nach den Eindrücken, die sie durch das eine Auge erhalten.

Diese Ergebnisse stehen in bestem Einklang untereinander und mit den Erfahrungen im sichtbaren Licht, wovon sie sich nicht unterscheiden. Danach sehen also die Süsswasserplanarien langwelliges ultraviolettes Licht. Sie nehmen es mit Hilfe ihres Hautlichtsinnes wahr, weit besser aber noch durch ihre Augen.

VIII. ZUSAMMENFASSUNG.

1. In allen bisher untersuchten Wirbeltieraugen (Säuger, Vögel, Reptilien, Amphibien) tritt eine starke bläulichweiße Fluoreszenz in UV Licht auf. Die am meisten strahlenden Augenlinsen zeigen Unterschiede im Leuchten. Jugendliche Linsen leuchten schwächer als ältere. Es scheint dass Dämmerungstiere weniger stark fluoreszierende Linsen haben als Tagtiere. Die Lichtdurchlässigkeit der Augenteile ist entsprechend der Fluoreszenz herabgesetzt, doch nicht völlig aufgehoben. Wirbeltiere sehen kaum Wellenlängen, die kürzer als $300\text{ m}\mu$ sind. Manche Linsen absorbieren Licht unterhalb $313\text{ m}\mu$ völlig (Frosch, Katze). Ein UV Bild auf der Netzhaut kann im Menschaugen schon durch Licht der Linsenfluoreszenz unterdrückt werden. Die Helligkeit des UV-Lichtes ist sehr gering. Darum stört uns das UV im Tageslicht nicht. Durch die kurzwelligen UV Sonnenstrahlen können in den vorderen Augenteilen Entzündungen hervorgerufen werden. Für gewöhnlich sind Netzhaut und Augengrund durch die starke Absorption der Strahlen in der Linse geschützt. Doch kann bei starkem UV-Licht die Netzhaut verletzt werden.

2. Auch im langwelligen UV beobachtet man bei Stichlingen eine Bewegung des Retinapigmentes. Zugleich löst es auch ein Wandern der Zapfen und Stäbchen aus. Im reinen UV sitzen die Zapfen auf der Grenzmembran auf, wie im sichtbaren Lichte auch. Fluoreszenzlicht zeigt die gleiche Wirkung, jedoch nicht so stark wie UV Licht.

3. Im Insektenauge entsteht ebenfalls überall da Fluoreszenzlicht wo farbloses Gewebe von UV Licht getroffen wird. Das farblose Chitin vor den Augen leuchtet bei genügender Dicke sehr stark. Trotzdem lässt sich UV-Licht auf dem Augengrund nachweisen. Die Tracheentapeta mancher Augen leuchten erheblich. An dem Verschwinden dieses Leuchtens erkennt man, dass auch dieses Augenpigment im UV bewegt wird. Bei empfindlichen Dämmerungsschmetterlingen vermag auch das Fluoreszenzlicht die Pigmentbewegung auszulösen, bei Tagesschmetterlingen hatte nur UV-Bestrahlung Erfolg.

4. Bertholf stellte für Bienen und Taufiegen fest, dass das Lichtspektrum im UV-Gebiet eine hohe Anziehungskraft besitzt, wenn man zugleich eine bestimmte Weisselligkeit bietet. Das stärkste Maximum liegt bei $366\text{ m}\mu$ und ist 4·5–5·5-mal so hoch wie das im grünen Bezirk. *Drosophila* hat drei Maxima. Eines bei $487\text{ m}\mu$, das hohe bei $366\text{ m}\mu$ und ein schwaches bei $254\text{ m}\mu$. Die Biene hat nur zwei Maxima, bei $555\text{ m}\mu$ und bei $366\text{ m}\mu$. Das Bienenspektrum ist im Rot länger und hört im kurzwelligen Gebiet bei $300\text{ m}\mu$ auf. Das *Drosophila*-Spektrum reicht über $300\text{ m}\mu$ hinaus, erscheint aber im Rot verkürzt.

5. Im langwelligen UV, das von unten kommt, legen sich die Daphnien wie im sichtbaren Licht auf den Rücken. Auch dann wenn man Fluoreszenzlicht zugleich von oben wirken lässt. Damit wird das Übergewicht des UV-Lichtes in seiner Wirkung auf die Daphnien bewiesen.

6. Planarien sind in langwelligem UV lichtflüchtig wie in sichtbarem Licht. Fluoreszenzlicht wirkt viel schwächer. Im Gegenlicht von 200–300 Lux wird der Treibwirkung von 0·07 Bunsen-Roscoe Einheiten UV das physiologische Gleich-

gewicht gehalten. Bei linksäugigen Planarien liegt das Fluchtfeld nach rechts, bei rechtsäugigen nach links. Die Zweiäugigen verfolgen ein sehr schmales Fluchtfeld symmetrisch nach vorn. Von oben belichtet, schlagen die linksäugigen Tiere Rechtskreise, die rechtsäugigen Linkskreise. Bei starkem Licht sind die Kreise, die das gleiche Tier zeichnet, kleiner als bei schwachem Licht. Die Planarien sehen also UV Licht.

IX. SUMMARY.

1. Ultra-violet light produces a strong bluish white fluorescence in the eyes of all vertebrate animals hitherto investigated (mammals, birds, reptiles, and amphibians). The lenses shine most strongly but to varying extents, those of young animals glowing less than the lenses of older individuals. It appears that twilight animals have less strongly fluorescent lenses than diurnal forms. The transparency of the component parts of the eye decreases in proportion to the amount of fluorescence, without, however, being completely abolished. Vertebrates can barely see wave lengths shorter than $300\text{ m}\mu$, and many lenses completely absorb light below $313\text{ m}\mu$ (frog, cat). Ultra-violet images on the human retina may be suppressed by fluorescence from the lens. The brightness of ultra-violet light is small, and for this reason it does not disturb us in daylight. Inflammation of the front parts of the eye may result from the shorter ultra-violet rays of the sun. Normally the retina and the floor of the eye are protected by the strong absorption of these rays in the lens. Nevertheless, strong ultra-violet light may injure the retina.

2. In minnows a movement of the retinal pigment occurs even in the long wave ultra-violet, accompanied by a migration of rods and cones. In pure ultra-violet the cones are situated on the limiting membrane, as they are in visible light. Fluorescent light produces the same effect, although less strongly than ultra-violet.

3. Fluorescence likewise results in the eyes of insects wherever ultra-violet light falls on colourless tissues. The colourless chitin over the eye shines strongly when it is sufficiently thick. Nevertheless, ultra-violet light cannot be demonstrated at the back of the eye. The tracheal tapetum of many eyes shines noticeably, and by the disappearance of the glow it can be recognised that this eye pigment too migrates in ultra-violet light. In the case of sensitive twilight butterflies fluorescent light can produce this movement of pigment, but in diurnal forms ultra-violet radiation alone is effective.

4. Bertholf showed that bees and *Drosophila* are attracted to the ultra-violet spectrum when they are exposed simultaneously to a certain intensity of white light. The strongest maximum is at $366\text{ m}\mu$ and is 4.5–5.5 times as strong as that in the green region. *Drosophila* has 3 maxima, one at $487\text{ m}\mu$, the highest at $366\text{ m}\mu$ and a weak maximum at $254\text{ m}\mu$. The bee has 2 maxima only, at $555\text{ m}\mu$ and $366\text{ m}\mu$. The bees' spectrum extends further into the red and ends in the short wave region at $300\text{ m}\mu$. The *Drosophila* spectrum extends beyond $300\text{ m}\mu$ but appears to be curtailed in the red.

5. *Daphnia* turns on to its back in long wave ultra-violet coming from below, just as it does in visible light, even when exposed at the same time to fluorescent light from above. This demonstrates the stronger effect of ultra-violet light on the animal.

6. Planarians move away from long wave ultra-violet just as they do from visible light. Fluorescent light has a weaker action. A physiological equilibrium is attained with a visible light of 200–300 lux opposed to 0.07 B.-R. units of ultra-violet. Left-eyed planarians move away from ultra-violet light to the right, right-eyed animals to the left, while those with both eyes move away along a very narrow track. Lighted from above the left-eyed animals circle to the right, those with right eyes the left. The circles are smaller in strong than in weak light. It follows that planarians can see ultra-violet light.

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CHEMICAL HETEROGONY AND THE GROUND-PLAN OF ANIMAL GROWTH¹

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(With Nineteen Text-figures.)

CONTENTS.

	PAGE
I. The concept of heterogony	79
II. Chemical heterogony	81
III. The logical status of the heterogony equation	82
IV. Changes in the heterogony constants	83
V. The convention of plotting	85
VI. Reproducibility	86
VII. Examples of the uniformity of chemical heterogony in different animals	87
VIII. Similarity of chemical heterogony in the same organ of different animals	97
IX. Effect of nutritive factors on chemical heterogony	100
X. Effect of temperature on chemical heterogony	100
XI. Heterogony and heat-production	102
XII. The chemical ground-plan of animal growth	104
XIII. Summary	107
References	108

I. THE CONCEPT OF HETEROGONY.

WHEN a living organism grows, its parts, as is well known, do not necessarily grow at exactly the same rate as the organism as a whole. Indeed a part of an organism which correctly reproduces the growth curve of the totality to which it belongs is the exception rather than the rule. "The form of an animal," as d'Arcy Thompson wrote in 1917, "is determined by its specific rate of growth in various directions; accordingly the phenomenon of rate of growth deserves to be studied as a necessary preliminary to the theoretical study of form, and mathematically speaking, organic form itself appears to us as a function of time." These facts have been well appreciated by the majority of morphologists, and a good deal of information about the growth of parts is now available (cf. the summaries of Scammon (1930), Fauré-

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Fremiet (1925) and Needham (1931)). If until recently it has remained somewhat unco-ordinated, this has no doubt been due to the inadequacy of the terminological and conceptual technique at our disposal. The work of Huxley (see especially his recent book (1932)) has, however, led the way towards a new order in this field.

There had been no lack of empirical equations, devised particularly in America, relating the weight of parts to that of the whole by a series of linear equations, in which many special constants were introduced. But in 1924 Huxley first demonstrated a simple and significant relation between the growth of a whole organism and that of a part increasing or decreasing in relative size. It had already been used, in closely similar form, by Dubois (1897) and Lapicque (1898 *a*) for the relative growth of the mammalian encephalon, but had remained hidden in the literature. If x be the magnitude of the whole organism (as measured by some standard linear unit, or by its weight), and y the magnitude of the differentially growing organ, the relation between them is

$$y = bx^k \quad \dots\dots(1),$$

where b and k are constants. The constant b is of little biological significance, since it merely denotes the value of y when x is unity, *i.e.* the fraction of x which y occupies when x is unity. It is called the "fractional coefficient." On the other hand, k has an important meaning, for it implies that over the range for which the formula holds, the ratio of the growth rate of the part to the growth rate of the whole remains constant. Such a ratio was termed by Huxley (1924) a "constant differential growth-ratio," and the whole process by Pézard (1918) "heterogony." Heterogony may obviously be either positive, if the relative size of the part increases with time (*i.e.* "grows more quickly than the whole"), or negative, if the relative size diminishes (*i.e.* "grows more slowly"). If it attains a very extreme degree, it may be called, in Champy's phrase (1924), "dysharmonic growth." Some authors, *e.g.* Teissier (1931), prefer to use the word "dysharmony" instead of "heterogony," but if we insist on borrowing terms from the technology of music, the growth of all the parts at the same rate as the whole would surely be "unison" and their growth at different rates "harmony" not "dysharmony." There is nothing pathological about relative growth.

The formula given above may also be written

$$\log y = \log b + k \log x \quad \dots\dots(2),$$

from which it follows that any magnitudes obeying the equation will fall along straight lines if plotted on a double logarithmic grid. The appearance of a straight line on double log paper does not establish the sufficiency of the formula for the case in question, since no purely graphical method could do so, but it affords strong evidence of its applicability. If the data were sufficiently good, each point could be verified by calculation. The logarithmic method of plotting, moreover, emphasises an important point which is entirely obscured by the usual method of plotting on absolute scales, namely, the fact that growth is essentially concerned with multiplication. Equal spaces on the logarithmic grid denote equal amounts of multiplication, equal spaces on the ordinary scale denote equal additions.

Another great advantage of logarithmic plotting consists in the fact that in embryonic development, where the numerical values on the axes cover such an enormous range, it is impossible in any other way to view the course of development as a whole in one and the same picture. For preliminary purposes, the constant k can be read off from the slope of the straight line, for if a is the angle it makes with the x axis

$$\tan a = k \qquad \dots\dots(3).$$

II. CHEMICAL HETEROGONY.

These methods were originally developed to deal with the phenomena of arthropod limb growth, the growth of antlers in some mammals, and other similar morphological magnitudes. But considerable scope exists for their application to magnitudes of a distinctively chemical nature. The metazoal organism, in its passage from fertilisation to maturity, passes through a long succession of stages which are characterised just as much by changing chemical composition as by changing morphological form. And just as we think of organs or structures as parts of a morphological totality, so we may think of substances or groups of substances as parts of a chemical totality. It is legitimate to enquire into the regularities which the constituents of this chemical totality exhibit during its continuing increase in mass. Now there exists in the literature of chemical embryology and the biochemistry of growth a large number of sets of data regarding such entities as protein nitrogen, glycogen, calcium, etc., in a variety of animals throughout the phyla. These may be considered from the point of view of heterogony. The constant k is here of great value, for it permits us to compare quantitatively the percentage relationships of any substance in any developing organism. A family of curves with meaningless shapes relating a chemical entity to age for several animals may become a series of straight lines of the same slope when the entity is logarithmically plotted against the totality. This is in fact the case, and arises because the complicating factor of time is not explicit in such graphs. The constant b , on the other hand, though it fixes the percentage composition of the body at unity, is not of great use, since the time at which unity is reached occurs at very different points in the life cycle owing to the great variation in the sizes of animals, and this is still true no matter what scale is taken.

Teissier (1931) was the first to apply heterogonic theory to the chemical development of an organism. Taking as experimental animals the larvae of the mealworm and the waxmoth, he studied the increase in a number of chemical entities, such as water, fat, ash, phosphorus, etc., and found that in all cases Huxley's relation was obeyed as the totality grew. In what follows, some of his results will be considered in more detail. The behaviour of the same substance in different classes of animals was, however, left over until Needham (1932 *a, b*), in papers mainly concerned with the evaluation of k for all the constituents of the chick embryo, drew attention to the rather close correspondence which may be exhibited by the relative "growth" of a given chemical entity in very different

animals. It is the purpose of the present paper to consider these cases and to bring forward a number of new ones.

III. THE LOGICAL STATUS OF THE HETEROGONY EQUATION.

Before going on to the experimental, or rather, observational data, reference must be made to the logical status of Huxley's equation. According to the Theory of Dimensions, the dimensions on the two sides of the relation must be equivalent. In this case,

$$y = bx^k \quad \dots\dots(1),$$

they are only so when k is 1, which is comparatively rare. Ordinarily, since x is a mass, x^k cannot be. The equation, therefore, has no true physical meaning, that is to say, no new concept can be deduced from it as it stands, in the sense that the concept of acceleration arises from the relation

$$Mf = a \quad \dots\dots(4),$$

between mass, force, and acceleration. The constant k is enigmatic without further elucidation, and cannot itself provide any theory of growth. The equation essentially gives us a good technical method for the examination of curves.

The point at issue may be made clearer by means of an analogy. The theory of dimensions is not satisfied in the relation

$$\Delta \propto c \quad \dots\dots(5),$$

where Δ is osmotic pressure and c is the concentration of a solute. Accordingly, until the implications in the relation are made explicit by considerations due to kinetic theory, the relation has no true physical meaning. A relation similar to the heterogony relation occurs in magnetism. If a mass of steel be carried through a cycle of flux from a maximum induction B to an equal negative and back again, the energy lost in such a cycle (Joules), per cubic centimetre of material, is

$$J = kf(B) \quad \dots\dots(6).$$

Now $f(B)$ has the same shape for all steel alloys, which differ solely in the value of k . Further, over a long range of B , $f(B) = c \cdot B^{1.6}$. Hence over that range

$$J = \eta B^{1.6} \quad \dots\dots(7).$$

Here there is no theoretical explanation of the exponent, and η is a constant varying with the alloy. The formula conveniently expresses relative losses, but a "magnetic theory" could not be founded on it, for it is purely an empirically verifiable numerical relation. Ultimately it arises from the fact that work has to be done to orient the particles of the iron. Similarly, in the heterogony equation for chemical entities, the ultimate explanation might be in terms of colloidal stability, but nothing can be premised concerning it from the equation, in the absence of further experiment and observation.

One attempt has already been made to offer a physical interpretation of the heterogony equation. Robb (1929) has suggested that the distribution of "building-stone" molecules between circulating blood and tissue cells proceeds in a similar

way to the distribution of solute molecules between two phases. Each organ would thus have a partition coefficient of its own for any given substance, and would grow more rapidly or more slowly than the body as a whole according to the value of this coefficient. Robb regarded the constant k as a measure of the concentration or activity of a substance governing the partition (*i.e.* capable of altering the partition coefficient), in so far as it was present in the organ in greater or less concentration than in the body as a whole. He also modified the Huxley equation by the insertion of a term, c , to represent the amounts of inert, non-growing substance present in the organ primordium; thus

$$y = bx^k + c \quad \dots\dots(8).$$

The requirements of the theory of dimensions do not seem to be any better satisfied here than in the original equation. This does not mean that the theory of partition coefficients is inapplicable, it only means that it does not follow from the empirical regularities so far found to hold. It fails, at once, of course, where chemical entities are concerned, for something more subtle is required to hold the balance between them. How far the introduction of a correction for non-growing constituents helps is not clear; so far practically all cases of chemical "growth" give linear plots on the double logarithmic grid.

IV. CHANGES IN THE HETEROGONY CONSTANTS.

Not all the sets of data for the relative growth of chemical constituents are representable by single linear plots; frequently two or even three separate straight lines may be found necessary. The breaks between these successive phases obviously indicate a change in one or other of the constants in the equation. If k changes, the slope of the line will alter; if b changes, there will be a period of nil growth of the part, followed by the resumption of growth at the same rate as before. These possibilities are exemplified in Fig. 1, which shows the water-soluble non-protein nitrogen of the chick embryo (Needham, 1927) plotted against the embryo's dry weight, and the water of the waxmoth larva (Teissier, 1931) plotted against the wet weight of the larva. The former entity grows with positive heterogony ($k = 1.69$) up to the sixth day of development, when the embryo weighs approximately 15 mg. (dry), after which it suddenly takes on a negative heterogony ($k = 0.83$). The latter entity (water in the waxmoth) grows throughout the whole period with a k of 0.96, but between the weights of 60 and 100 mg. (wet) there is a period of adjustment during which b changes from 0.0075 to 0.0055.

If the results of Teissier for the insect larvae are compared with those of Needham for the chick embryo, it is at once noticeable that changes of the first type (changes in k) are much more characteristic of the embryonic material than those of the second type, and *vice versa*, although a few examples of the opposite correlation can be found. Any interpretation of this fact might as yet be premature, but it may not unreasonably be connected with the circumstance that the larval insect growth is much less accompanied by profound changes of morphological differentia-

tion than is the growth of the chick embryo. Further examples are needed to test this possibility. For the present purpose, however, discussion will be mainly confined to those cases where the data exhibit no breaks, no changes in either b or k . This is not because the breaks may not be perfectly real, but because greater

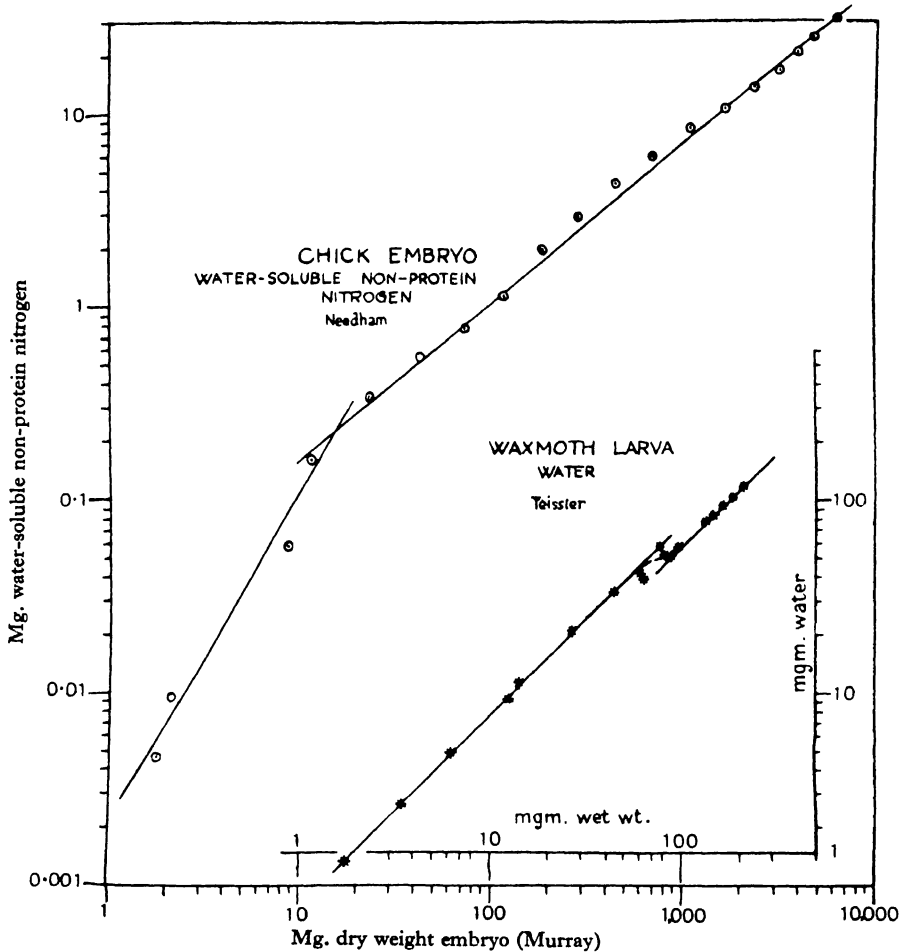


Fig. 1. Examples of change in b and change in k ; for the former, water in the waxmoth larva (Teissier, 1931); for the latter, non-protein nitrogen in the chick embryo (Needham, 1927).

certainty of comparison between different organisms may be attained by the use of data which do not show them. For example, the calcium in the chick embryo has been determined by several investigators (references and plots in Needham, 1932 *b*), some of whose series give breaks of k . But when the entire mass of data is plotted without distinction of investigator, it is found to lie with equal deviation on both sides of a straight line from the smallest to the very largest embryos. This

suggests that with further work, many of the changes in k hitherto recorded may turn out to be illusory. But in the present paper, emphasis will only be laid on sets of data giving the unbroken logarithmic relationship, and on its similarity between different animals.

V. THE CONVENTION OF PLOTTING.

In the following graphs it will be seen that in all cases the mass of the chemical entity is plotted against the mass of the chemical totality. Although this is quite logical, it implies that in the case of a part growing with positive heterogony, *i.e.* faster than the whole, a time must eventually come when the mass of the part equals that of the whole, and finally exceeds it. This unfortunate state of affairs is avoided by writing the equation

$$y = b(x - y)^k \quad \dots\dots(9),$$

and plotting the part against the remainder of the whole rather than the whole itself. This was the method adopted by Huxley (1924) in the first instance, who defined x accordingly, although the graphs in his book (1932) do not seem to be always

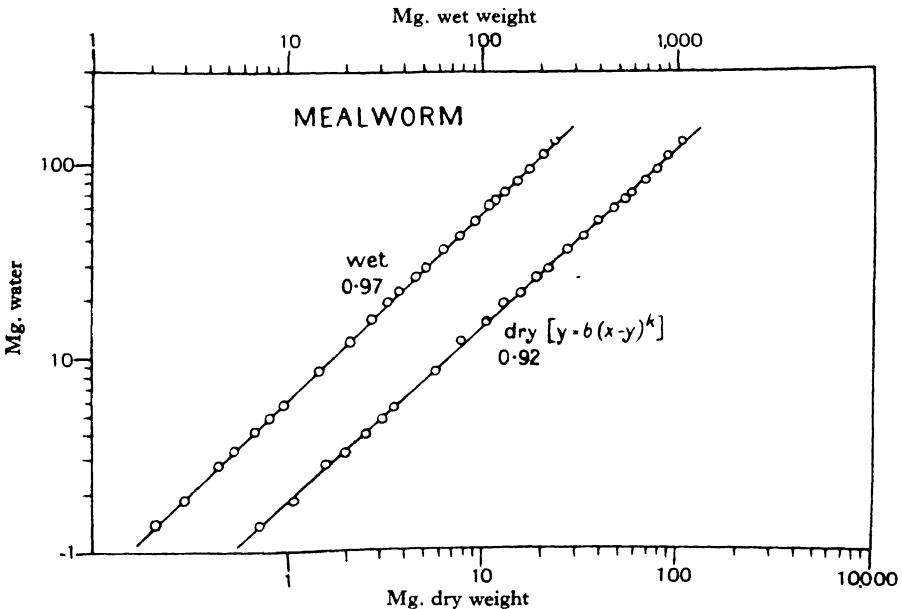


Fig. 2. Comparison of the relation of water in the mealworm larva to wet weight and dry weight (Teissier, 1931).

consistent with it. Teissier (1931), on the other hand, used the formula (1), and its application to chemical heterogony will be continued here. The question is somewhat academic, since growth is not infinite, and a point imagined as travelling along one of the straight lines in the succeeding figures would slow down and finally stop at the adult condition long before the consequences of the convention

here adopted became serious. Moreover, the difference in k is not very marked for the two conventions. In the case of a very small part it is quite inappreciable, and even in that of a very large part, such as the water content of the body, it is comparatively small. Fig. 2 shows the water in the body of the mealworm larva, plotted against the wet weight (x) and the dry weight ($x-y$) respectively. In the former case k is 0.97, in the latter case 0.92.

VI. REPRODUCIBILITY.

A word should here be said concerning the reproducibility of graphs of chemical heterogony. It seems to be satisfactory. In Fig. 3 the solid line represents the

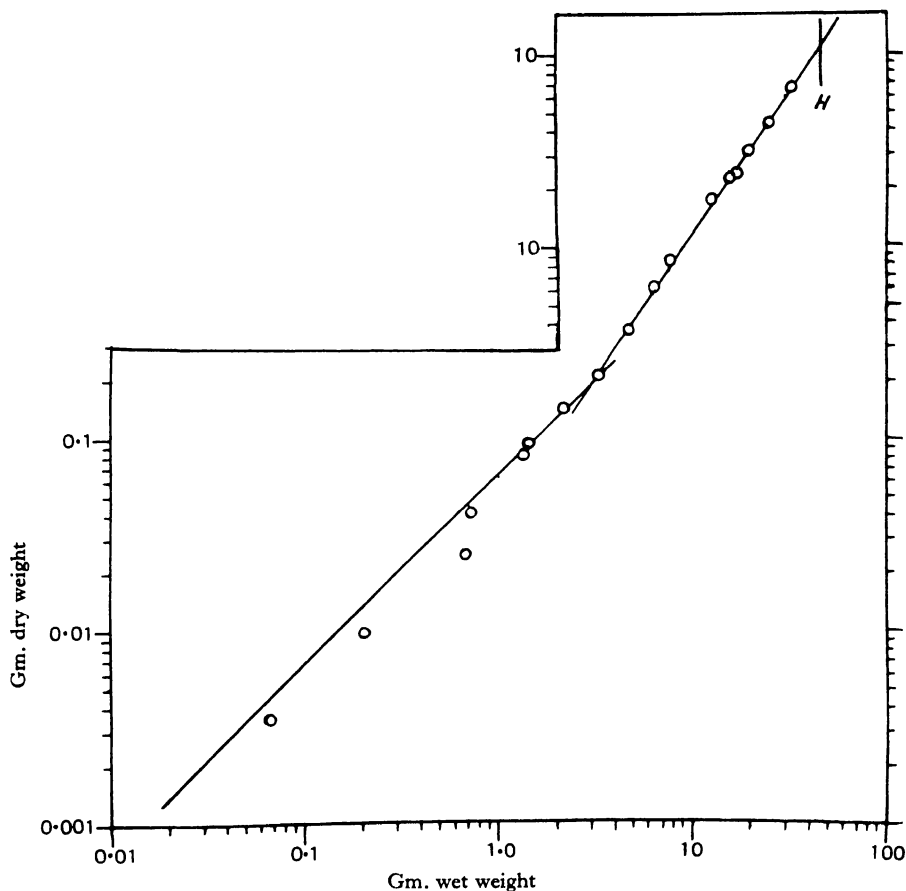


Fig. 3. Relation of dry to wet weight in the chick; confirmation of earlier workers by Saccardi and Latini (1931).

relation of dry weight to wet weight in the chick embryo as determined from the averaged data of thirteen separate investigators (see Fig. 220 in Needham, 1931).

The circles represent the data recently reported by Saccardi and Latini (1931), who appear to have been unaware of the work of their predecessors. It will be seen that over the greater part of the range the agreement is excellent, although in the earliest stages, when the embryos are very small, the Italian workers show a slight divergence.

VII. EXAMPLES OF THE UNIFORMITY OF CHEMICAL HETEROGONY IN DIFFERENT ANIMALS.

We may now pass to specific examples. It may be convenient first to consider water content. Fig. 3 showed that during the first period in the development of the chick embryo, the increase in solids is practically isogonic, and during the second period strongly positively heterogonic ($k = 0.93-1.06$, then $1.43-1.48$). This is only a more accurate way of stating the well-established fact that the chick embryo becomes drier as it develops. For organisms other than birds, numerous sets of data exist covering in some cases the whole life cycle, in others only pre-natal or only post-natal life. A good many of these are plotted in Fig. 4 on one double logarithmic grid, and the references and constants are collected in Table I. The points of birth or hatching are suitably marked on the chart in each case.

It is at once obvious that within any one group of animals there is a marked similarity of slope, that is to say, they get drier at the same absolute rate. The mammals, for instance, all show a k closely approximating to 1.23, and it is remarkable that over the whole growth period of man (from 1 to 66,000 gm. wet weight) this relationship holds good. Again, the three researches on teleostean fishes give a k of unity, indicating that over the period studied (from some time before hatching until the end of the free-swimming larval period) the tissues are not drying at all. The same statement applies to the woodlouse, which, however, has only been measured (and that some considerable time ago) after hatching. Critical points, where k changes, appear both in the chick, and in some of the selachian fishes, which show the same change from isogonic growth of the dry weight to marked positive heterogonic growth. On the whole, it may be concluded that dry weight never shows negative heterogony, that is to say, all organisms dry up with age, and that within one group the absolute rate at which this process takes place is very uniform indeed. In Table I the ranges of k are given as well as the average values. The birds alone show a wide range, but this may be explained by the existence of two separate phases, and the restricted nature of some of the sets of data available. The range of all k 's for all organisms is only 0.27.

In those cases where no drying up of the organism is taking place, we have not as yet sufficient knowledge of the dry weight throughout the life cycle to say whether the process of dehydration has finished or whether it has not yet begun. It is tempting to relate the concurrence of evidence for the isogony of the teleostean fishes with the views of Bidder (1925, 1932) on the absence of adult size in some aquatic organisms, and the possible absence of death from senility. Although plaice and carp, he suggests, may continue to grow until many times the age of their

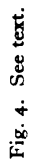


Fig. 4. See text.

sexual maturity, and although we have no reason to suppose that they ever die, except by violence, the same could not be true of swiftly moving terrestrial creatures. These must maintain a relation between their weight and the cross-sectional area of their bones and muscles, necessitating a definite adult size, shape, and habit. Old age and death by senility became the necessary fate for animals only when they left the water and attempted swiftness and tallness in a medium $1/800$ th of their own specific gravity. On the other hand, there is at present no obvious reason why the tissues of selachian fishes should dehydrate with growth, while those of teleosts do not. The subject merits much closer attention than it has yet received.

The next example which may be taken is that of fat. Fig. 5 gives the appearance of the data for rat, chick, and mealworm when plotted on the double logarithmic

Table I. *Dry weight as function of wet weight.*

Organism	Investigators	Period	k	Range of k	Average k
MAMMALS					
Man (<i>Homo sapiens</i>)	Michel; Fehling; Brubacher; Camerer and Soldner; Langstein and Edelstein; Sommerfeld; Moleschott; Bischoff; collected by Aron (1928), also Schmitz (1923) and von Bezold (1857, 1858)	Whole life	1.26	0.07	1.23
Rabbit (<i>Lepus curvulus</i>)	Fehling; Schkarin; Friedenthal; Steinitz; collected by Aron (1928)	Whole life	1.28		
Dog (<i>Canis vulgaris</i>)	Thomas; Gerhardt; Orgler; Eckert; collected by Aron (1928)	Whole life	1.24		
Cat (<i>Felis vulgaris</i>)	Thomas (1911)	Post-natal life	1.21		
Kat (<i>Mus norvegicus</i>)	Lowrey, Donaldson; collected by Donaldson (1924) also y Gonzalez (1932)	Whole life	1.23		
Mouse (<i>Mus vulgaris</i>)	von Bezold (1857, 1858)	Whole life	1.21		
BIRDS					
Fowl (<i>Gallus domesticus</i>)	Murray; Romanov; Byerly; Man-kin; collected by Needham (1931)	Embryonic life	1.00 then 1.42	0.30	1.19
Pigeon (<i>Columba livia</i>)	Kaufman (1926)	Post-embryonic life	1.19		
Thrush (<i>Turdus merula</i>)	Groebbels (1927)	Embryonic life	1.03		
Crow (<i>Corvus corone</i>)	Groebbels (1927)	Embryonic life	1.04		
Hawk (<i>Buteo buteo</i>)	Groebbels (1927)	Embryonic life	1.28		
REPTILES					
Lizard (<i>Lacerta viridis</i>)	von Bezold (1857, 1858)	Post-embryonic life	1.11	0.09	1.15
Turtle (<i>Thalassochelys corticata</i>)	Karashima (1929)	Embryonic life	1.20		
AMPHIBIA					
Frog (<i>Rana temporaria</i>)	von Bezold (1857, 1858)	Post-larval life	1.07	0.08	1.09
(<i>Bombinator igneus</i>)	von Bezold (1857, 1858)	Post-larval life	1.15		
Triton (<i>Triton cristatus</i>)	von Bezold (1857, 1858)	Post-larval life	1.07		
TELEOSTEAN FISHES					
Salmon (<i>Salmo salar</i>)	Hayes (1930)	Embryonic and larval life	1.00	0.0	1.00
Trout (<i>Salmo fario</i>)	Gray (1926)	Embryonic and larval life	1.00		
	Kronfeld and Scheminzki (1926)	Embryonic and larval life	1.00		
		Embryonic and larval life	1.00		
SELACHIAN FISHES					
Dogfish (<i>Scyllium canicula</i>)	Ranzi (1932)	Embryonic life	1.21	0.12	1.27
Torpedo (<i>Torpedo ocellata</i>)	Ranzi (1932)	Embryonic life	1.00 then 1.33		
Torpedo (<i>Torpedo marmorata</i>)	Ranzi (1932)	Embryonic life	1.00 then 1.32		
Dogfish (<i>Acanthias blanchardi</i>)	Ranzi (1932)	Embryonic life	1.00 then 1.29		
Dogfish (<i>Mustelus vulgaris</i>)	Ranzi (1932)	Embryonic life	1.00 then 1.23		
CEPHALOPOD					
Squid (<i>Sepia officinalis</i>)	Ranzi (1930)	Embryonic life	1.09	—	1.09
INSECTS					
Mealworm (<i>Tenebrio molitor</i>)	Teissier (1931)	Larval life	1.04	0.01	1.035
Waxmoth (<i>Galleria mellonella</i>)	Teissier (1931)	Larval life	1.03		
CRUSTACEA (terrestrial)					
Woodlouse (<i>Oniscus asper</i>)	von Bezold (1857, 1858)	Post-larval life	1.00	—	1.00

Note. The recent data of Wilkerson and Gortner (1932) for the pig are not included for reasons of technique; cf. also Esskuchen (1931) for the cow.

grid (data of Chanutin (1931), Murray (1926), Cahn (1928), Romanov (1932) and Teissier (1931)). For the insect and the bird, in spite of the great differences in the absolute weights concerned (*e.g.* at hatching or birth, mealworm = 3 mg. dry weight, chick = 7000 mg. dry weight, rat = 660 mg.), the correspondence is very good; the rat shows a slightly more positive heterogony. The values of *k*, together with their range of variation, are shown in Table II. In all cases, the heterogony

Table II. *Chemical heterogony in various organisms.*

Fig.	Chemical substances or group of substances	Animal	Investigators	Period	<i>k</i>	Range of <i>k</i>	Average <i>k</i>
5	Fat (to dry weight)	Mealworm	Teissier (1931)	Larval	1.07	0.03	1.07
		Chick	Murray (1926); Cahn (1928); Romanov (1932)	Embryonic	1.06		
6	Creatine (to wet weight)	Rat	Chanutin (1931)	Post-natal	1.09	0.02	1.11
		Chick	Mellanby (1907)	Embryonic	1.12		
7	Glutathione (to wet weight)	Rat	Chanutin (1931)	Post-natal	1.10	0.01	0.845
		Pig	Thompson and Voegtlin (1926)	Whole life	0.84		
8	Glycogen (to wet weight)	Pig	Wilkerson and Gortner (1932)	Embryonic	0.85	0.07	1.37
		Chick	Vladimirov and Danilina (1930)	Embryonic	1.42		
9	Liver glycogen (to wet weight of liver)	Pig	Mendel and Leavenworth (1908)	Embryonic	1.35	0.03	1.975
		Rabbit	Lochhead and Cramer (1908)	Embryonic	1.35		
10	Total ash (to dry weight)	Chick	Vladimirov (1930)	Embryonic	1.99	0.03	1.975
		Rabbit	Lochhead and Cramer (1908)	Embryonic	1.06		
11	Calcium (to dry weight)	Mealworm	Teissier (1931)	Larval	0.82	0.14	0.90
		Squid	Ranzi (1930)	Embryonic	0.86		
		Dogfishes	Ranzi (1932):				
			<i>Scyllium canicula</i>	Embryonic	0.96		
			<i>Torpedo marmorata</i>	Embryonic	0.96		
			<i>Torpedo ocellata</i>	Embryonic	0.86		
			<i>Mustelus vulgaris</i>	Embryonic	0.85		
		Chick	Murray (1926)	Embryonic	0.89		
			Romanov (1929)	Embryonic	0.89		
			Bishop (1929)	Embryonic	0.85		
			Saccardi and Latini (1931)	Embryonic	0.94		
		Rat	Chanutin (1931)	Post-natal	0.95		
12	Total phosphorus (to dry weight)		Buckner and Peter (1922)	Post-natal	0.93	0.08	1.18
		Chick	Mankin (1929, 1930); Plummer and Lowndes (1924); Romanov (1930)	Embryonic	1.21		
13	Lead (to dry weight)	Rat	Sherman and McLeod (1925)	Post-natal	1.13	0.05	0.97
		Man	Schmitz (1923)	Pre-natal	1.20		
14	Fat (to wet weight)	Mealworm	Teissier (1931)	Larval	0.97	0.05	0.97
		Waxmoth	Teissier (1931)	Larval	0.95		
15	Fat (to wet weight)	Rat	Buckner and Peter (1922); Sherman and Quinn (1926)	Post-natal	1.00	0.05	0.97
		Chick	Bishop (1929)	Embryonic	1.00		
16	Fat (to wet weight)			High	0.93	0.05	0.97
				Low	0.93		
17	Fat (to wet weight)	Chick	Romanov and Faber (1933)	Embryonic	1.84	0.05	0.97
				32° C.	1.85		
18	Heat-production (to live weight)			34° C.	1.77	0.19	0.61
				36° C.	1.79		
19	Heat-production (to live weight)			38° C.	1.79	0.19	0.61
					1.79		
20	Heat-production (to live weight)	Chick	Brody <i>et al.</i> (1932)	Post-natal	0.69	0.19	0.61
		Rabbit	Brody <i>et al.</i> (1932)	Post-natal	0.61		
21	Heat-production (to live weight)	Guinea-pig	Brody <i>et al.</i> (1932)	Post-natal	0.60	0.19	0.61
		Rat	Brody <i>et al.</i> (1932)	Post-natal	0.57		
22	Heat-production (to live weight)	Dog	Brody <i>et al.</i> (1932)	Post-natal	0.65	0.19	0.61
		Sheep	Brody <i>et al.</i> (1932)	Post-natal	0.55		
23	Heat-production (to live weight)	Pig	Brody <i>et al.</i> (1932)	Post-natal	0.70	0.19	0.61
		Cow	Brody <i>et al.</i> (1932)	Post-natal	0.62		
24	Heat-production (to live weight)	Horse	Brody <i>et al.</i> (1932)	Post-natal	0.51	0.19	0.61
		Man	Brody <i>et al.</i> (1932)	Post-natal	0.59		

is positive, that is to say, the fat is increasingly present in the body with growth. Other aspects of this phenomenon are often met with, *e.g.* the development of mutton quality in the sheep (Hammond and Appleton, 1932) and the chemical development of the mammalian brain, to be referred to later.

Next, two important tissue extractives, creatine and glutathione, are illustrated

in Figs. 6 and 7. The positive heterogony of creatine in the chick shown by Mellanby's (unfortunately rather restricted) data (1907) is exactly paralleled by that of the same substance in the rat according to Chanutin (1931). Similarly, the marked negative heterogony of glutathione is identical for the rat and the pig (data of Thompson and Voegtlin (1926) and Wilkerson and Gortner (1932) respectively). Creatine, then, "grows" more rapidly than the whole body, glutathione more slowly. The range covered by the data in Fig. 7 is very wide, in the case of the rat from 0.2 to 200 gm. wet weight, in the case of the pig from 1.0 to 1000 gm. wet weight.

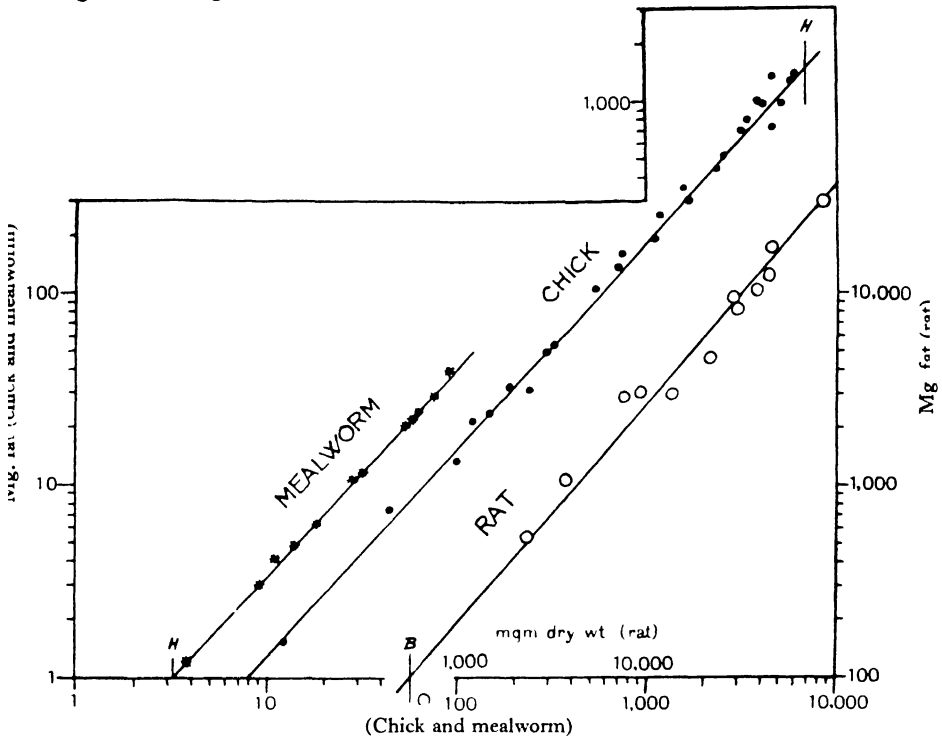


Fig. 5. Fat in the mealworm (larval period: Teissier, 1931), the chick (embryonic period: Murray, 1926; Cahn, 1928; Romanov, 1930), and the rat (post-natal life: Chanutin, 1931).

Glycogen is a substance which has a rather special relation to embryonic life, since until the liver is sufficiently developed to store it, it is laid down in the extra-embryonic structures (mammalian placenta, avian yolk-sac) which perform the functions of a transitory liver (see Needham, 1931, section 8.5). Nevertheless there is remarkable regularity between different animals in the rates at which the substance increases in the embryonic body itself. Fig. 8 shows the data for the chick, rabbit, and pig, all of which give a k in the near neighbourhood of 1.38. Even when we consider the accumulation of glycogen in the embryonic liver, we find that $k = 1.97$ would hold for both chick and rabbit—a remarkable circumstance

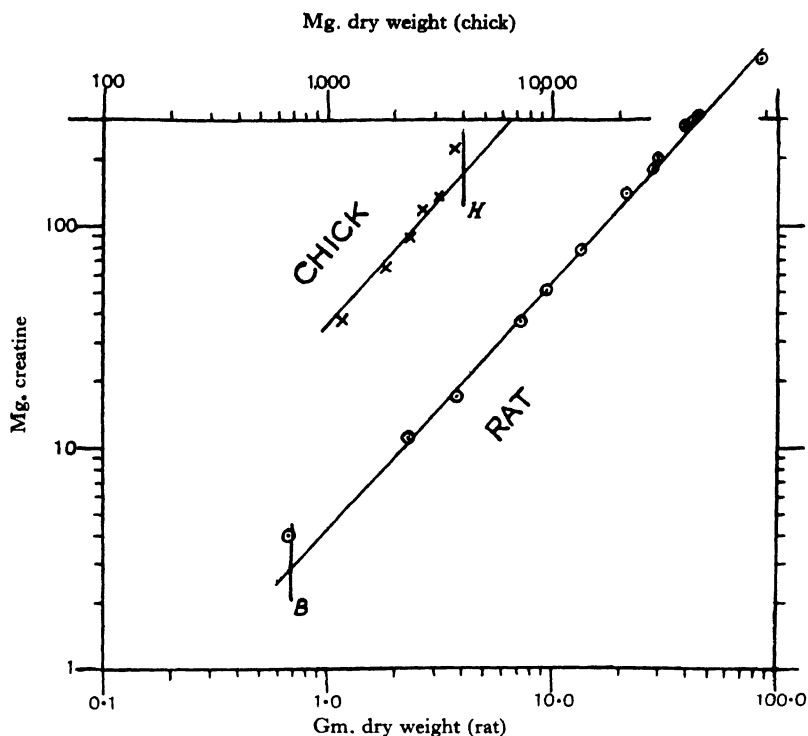


Fig. 6. Creatine in the chick embryo (Mellanby, 1907) and in the rat (post-natal life: Chanutin, 1931).

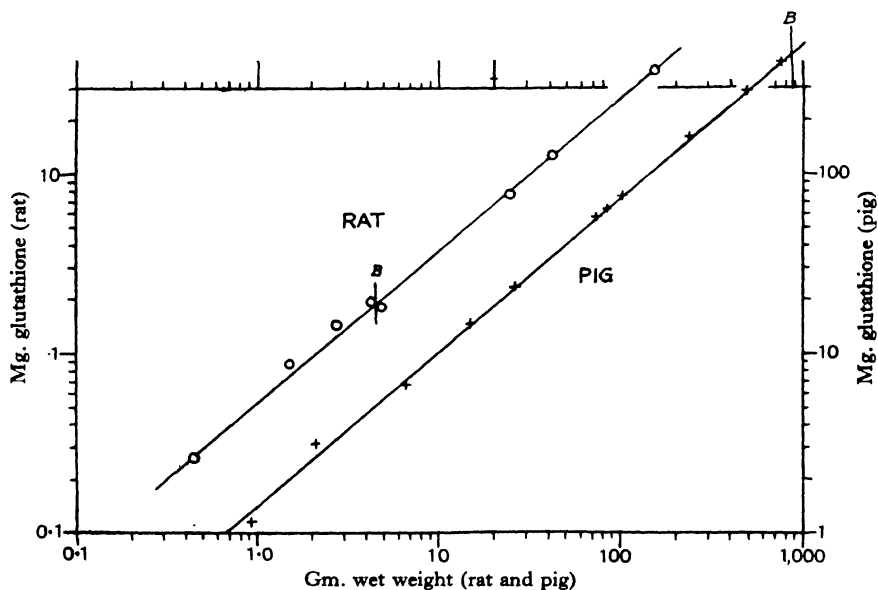


Fig. 7. Glutathione in the rat embryo (Thompson and Voegtlin, 1926) and in the pig embryo (Wilkerson and Gortner, 1932).

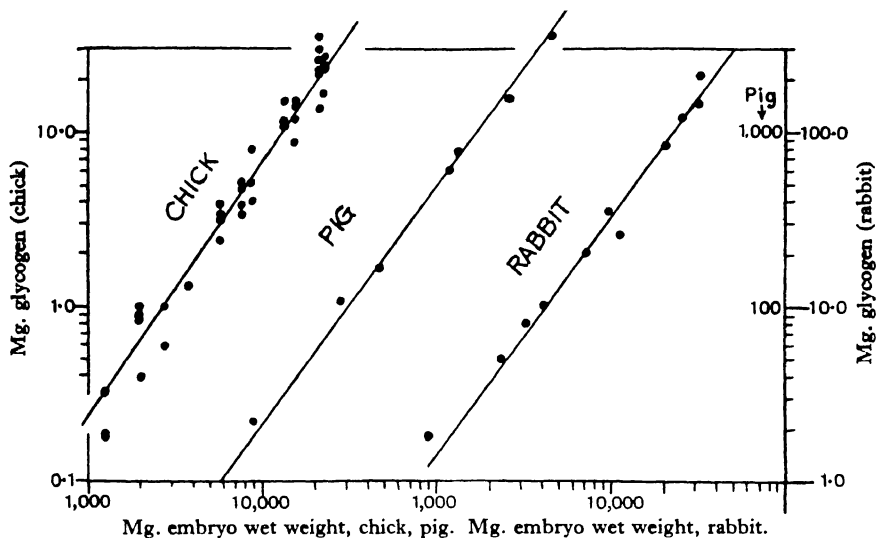


Fig. 8. Glycogen in the chick embryo (Vladimirov and Danilina, 1930), in the pig embryo (Mendel and Leavenworth, 1908), and in the rabbit embryo (Lochhead and Cramer, 1908).

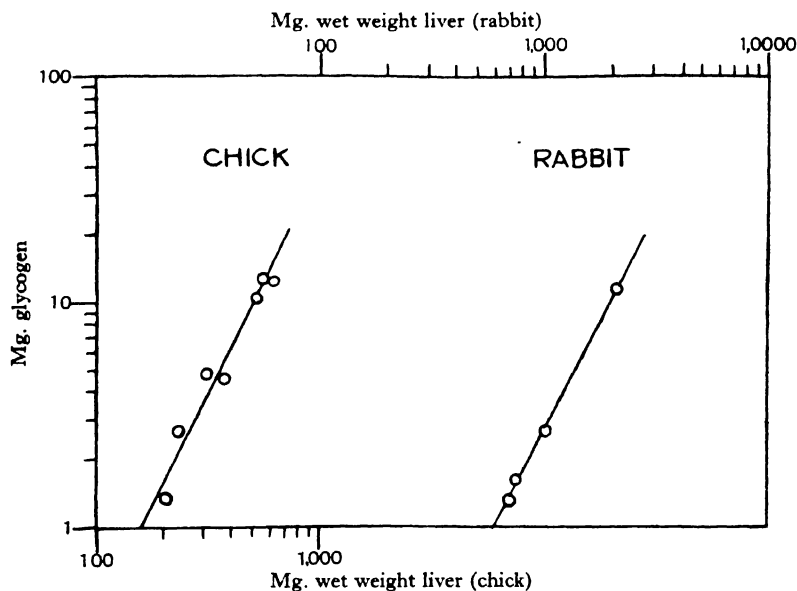


Fig. 9. Glycogen in the liver of the chick embryo (Vladimirov, 1930) and in the liver of the rabbit embryo (Lochhead and Cramer, 1908).

when we remember that structures so different as the avian yolk-sac and the rodent placenta are handing over glycogen to the foetal liver after serving as temporary dépôts themselves.

The next graph (Fig. 10) is a particularly interesting one. The total ash of the body is plotted against the dry weight for the chick, rat, squid, mealworm, and several selachian fishes. The chick, the squid, and the fishes are all embryonic, the mealworm is larval, and the rat is post-natal. There is a close uniformity in the slopes of the straight lines, in spite of the enormous differences of morphological form and of time taken to accomplish the changes depicted. Thus the chick series is accomplished in three weeks, the development of the squid and the selachian fishes may take several months, and that of the rat more than a year. Again, the absolute values vary from a few milligrammes in the mealworm to kilos in the selachian fishes. Finally, the nutritive factors are quite different (Needham, 1931). The chick finds all the ash which it requires for its body in the egg interior and the shell, but the embryo of the squid has to absorb 30 per cent. of the inorganic substances required from the sea water surrounding its egg. As for the selachian fishes, *Scyllium* develops in a "closed box" but nevertheless absorbs 75 per cent. of its ash from the sea water, while the others, developing ovoviviparously within the maternal body (but without placentoid attachments), absorb 25, 60 and 97 per cent. respectively of their ash from the maternal blood stream. The rat and the mealworm receive the ash in their food in the ordinary way. But in spite of these far-reaching nutritive differences, the heterogony is uniformly negative, as Table II demonstrates.

Among the constituents of the ash, calcium and phosphorus may be selected for examination. Fig. 11 shows the calcium in the human embryo (Schmitz, 1923), the chick embryo (Mankin, 1929, 1930; Romanov, 1930; and Plimmer and Lowndes, 1924), and the rat (Sherman and McLeod, 1925). In all three series of data a closely similar slope is observable, showing that with increasing size the body contains relatively larger quantities of the metal. Thus from 0.01 to 10.0 gm. dry weight in the case of the chick embryo, and from 0.2 to 100.0 gm. dry weight in the case of the human embryo, the absolute increase rate of calcium is the same. The data of Wehefritz (1925) for the calcium in the human placenta have been included on the same graph, but the points are too scattered and too limited in locality to make the drawing of a straight line through them justifiable. It can be seen, however, that a line drawn parallel to the embryo line does not greatly misrepresent them, and it is quite possible that the growth of the placenta follows a similar course to that of other cells of embryonic origin. We have here the requisite conception for a new analysis of the constitution of the placenta.

The period of life covered in Fig. 11 is that during which ossification is proceeding, and at first sight it may appear strange that the onset of the process is not accompanied by profound changes in the differential growth ratio. The data clearly indicate, however, that the deposition of calcium in the bones is essentially a matter of translocation of the element, and does not involve any increase of intake at the same time.

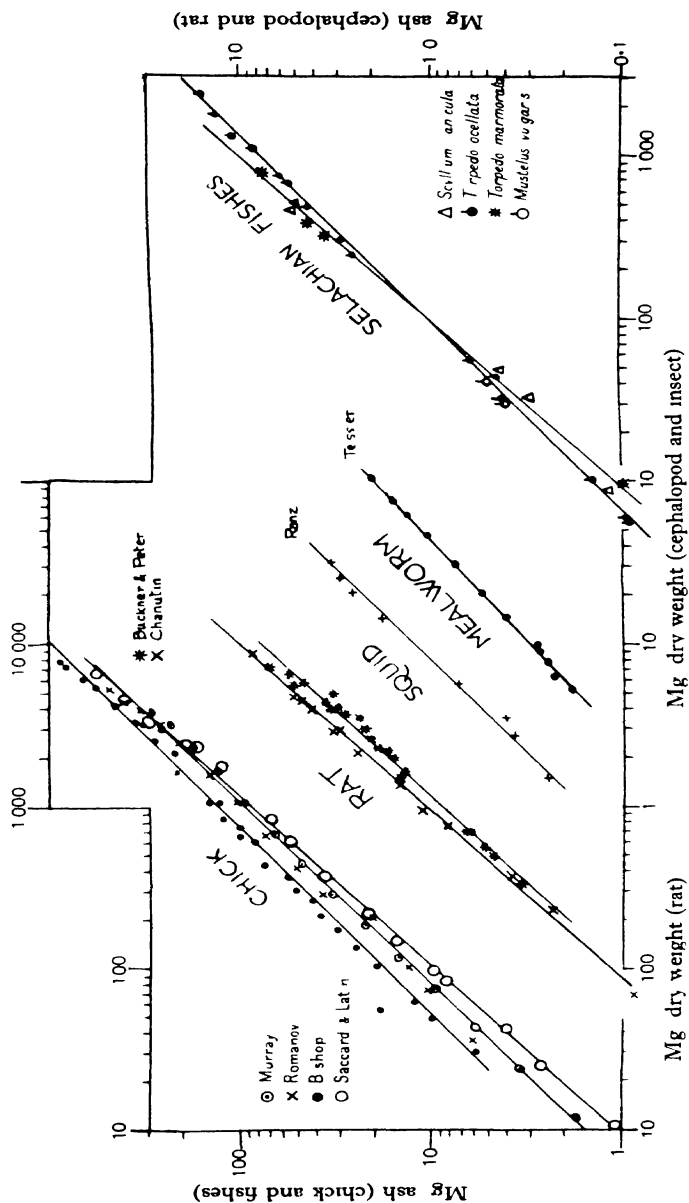


Fig 10 Ash in the chick embryo (Murray, 1926, Romanov, 1930, Bishop, 1929, Saccardi and Latini, 1932), in the rat (post-natal life Chanutin, 1931, Buckner and Peter, 1922), in the squid embryo (Ranzi, 1930), the mealworm (larval life Teissier, 1931), and various selachian fish embryos (Ranzi, 1932)

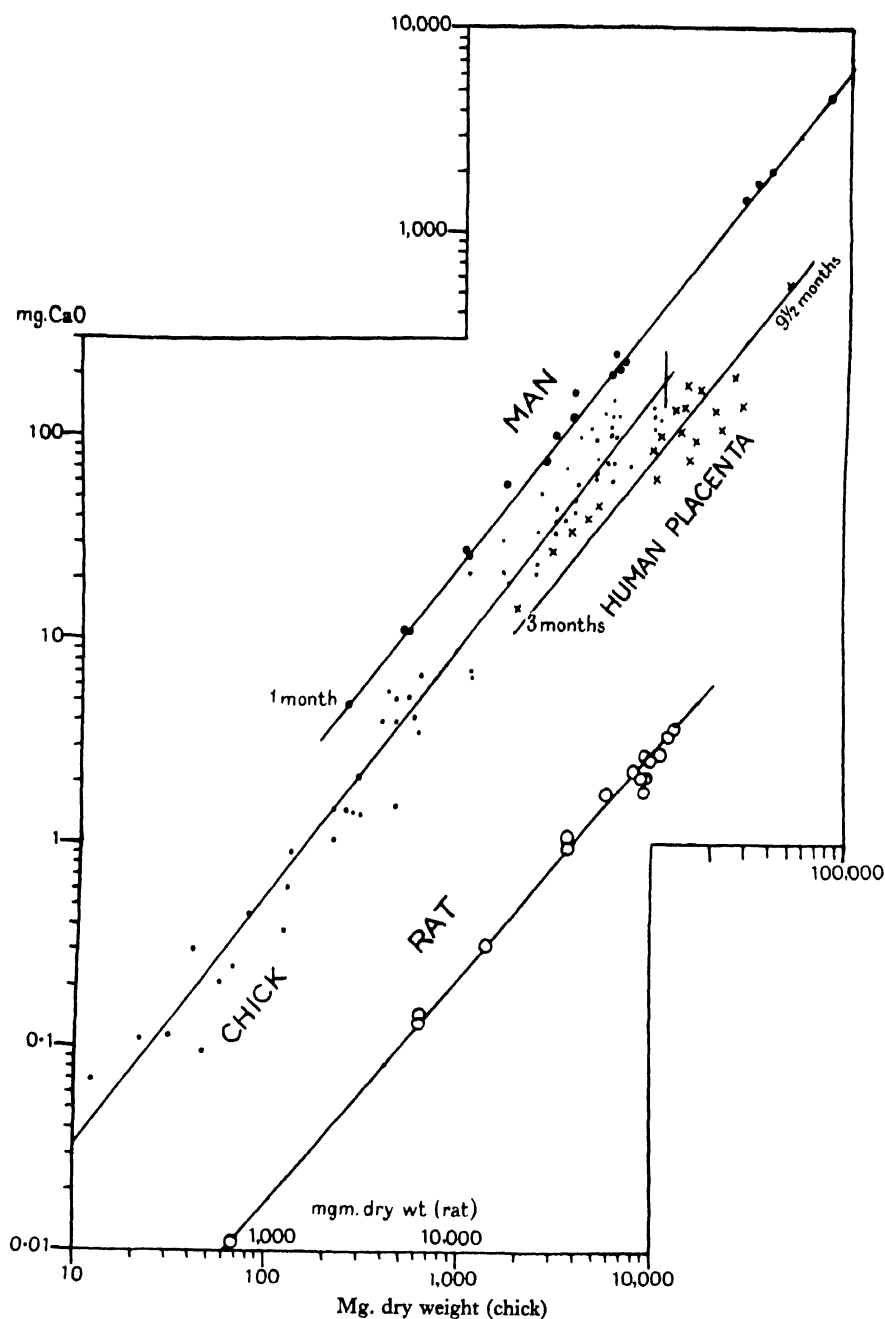


Fig. 11. Calcium in the chick embryo (Mankin, 1929, 1930; Plimmer and Lowndes, 1924; Romanov, 1929), in the human embryo (Schmitz, 1923), and in the rat (post-natal life: Sherman and McLeod, 1925).

Unlike calcium, phosphorus is an element which shows an isogonic relation. The data for the post-natal life of the rat (Buckner and Peter, 1922; Sherman and Quinn, 1926) and for the larval life of the mealworm and waxmoth (Teissier, 1931), shown in Fig. 12, illustrate this clearly. If any heterogony is present at all, it is

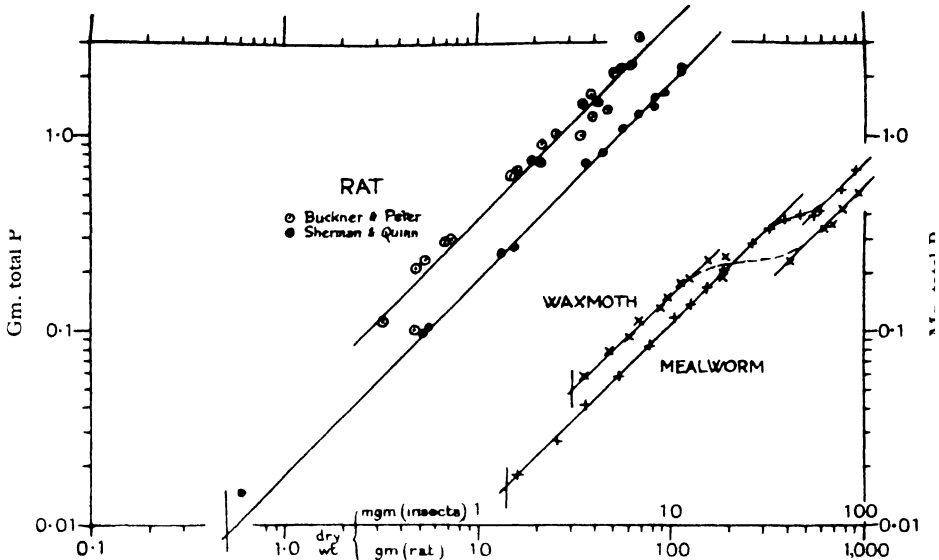


Fig. 12. Total phosphorus in the rat (post-natal life: Buckner and Peter, 1922; Sherman and Quinn, 1926), in the mealworm and in the waxmoth (larval life: Teissier, 1931).

very slightly negative. The insect data here show changes in the constant b , as referred to above. A biochemical interpretation of this behaviour is difficult, especially as total phosphorus includes so many separate fractions (lipoid P, nuclein P, inorganic P, etc.). The interest lies in the close similarity of the mammal and the insects.

VIII. SIMILARITY OF CHEMICAL HETEROGONY IN THE SAME ORGAN OF DIFFERENT ANIMALS.

So far (except for liver glycogen) we have been discussing the changes occurring in the body of the organism as a whole. It is interesting to examine the development of individual organs and tissues, and of these a good instance for the present purpose is the mammalian brain¹. Donaldson and Hatai (1911) studied the water content of the developing brain of the rat, while Koch and Koch (1913) and McArthur and Doisy (1919) investigated that of man, and Bäcklin (1930) that of the rabbit. In Fig. 13 the dry solid in the brain of rat and man is plotted against the wet weight.

¹ It is of interest that as long ago as 1898 Lapicque (1898 *b*) compared the ether extracts of brains of different sizes, taken from a series of mammals. The available technique, however, was too crude to permit of any sure conclusions.

The correspondence between the slopes of the straight lines resulting is remarkably good ($k=1.38$ for the rat, 1.33 for man).

The correspondence between the general pictures of constituents for the mammalian brains is, however, equally striking. Fig. 14 gives the data for proteins, phosphatides, cerebroside, sulphatides, organic and inorganic extractives, and cholesterol. It can be seen at a glance that although the general level of absolute weight is quite different, and the time of occurrence of birth is also different, the principal course of events is closely similar. The slopes agree, and each constituent increases relatively at a characteristic pace. The increase continues so long as the brain is growing except in the case of the organic extractives and ash, a fraction

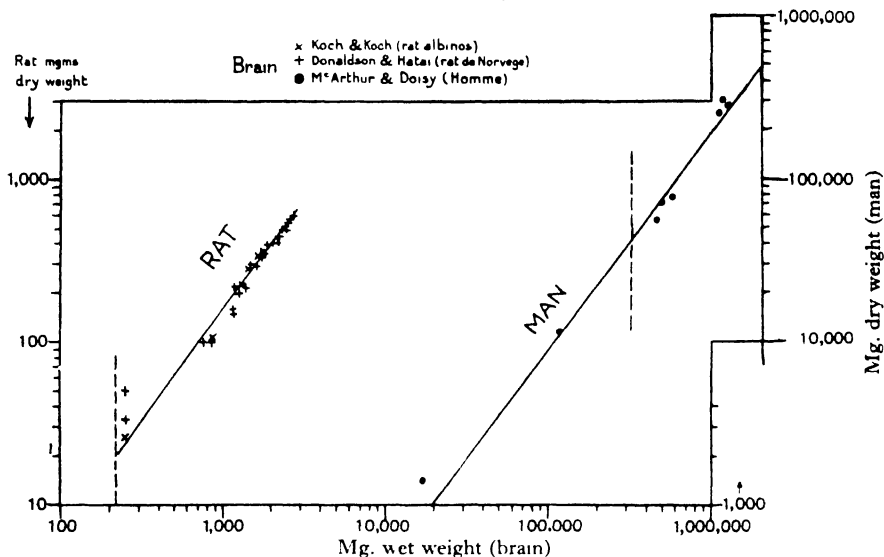


Fig. 13. Dehydration of the brain of the rat (post-natal life: Koch and Koch, 1913; Donaldson and Hatai, 1911) and of the brain of man (pre- and post-natal life: McArthur and Doisy, 1919). The vertical dotted lines indicate birth.

which seems to come to completion at a certain point, and thereafter to increase no more. Particularly interesting is the very rapid increase of the cerebroside, where sometimes two phases seem to be indicated; it occurs last of all and is certainly associated with myelinisation. The numerical values and ranges of k are shown in Table III.

From Table III we find that the proteins and the organic and inorganic extractives show definite negative heterogony, while the phosphatides, cerebroside, and sulphatides, together with the sterols, show pronounced positive heterogony. This is equivalent to saying that the structure is first laid down on a protein (one might almost say water-soluble) basis, and it is only later on that the lipid and sterol molecules come to take up their places in the nervous system. This is just what we see in the chick embryo, for the histochemical work of Marza (1929) has shown that in the nervous system at the primitive streak and somite stage, there

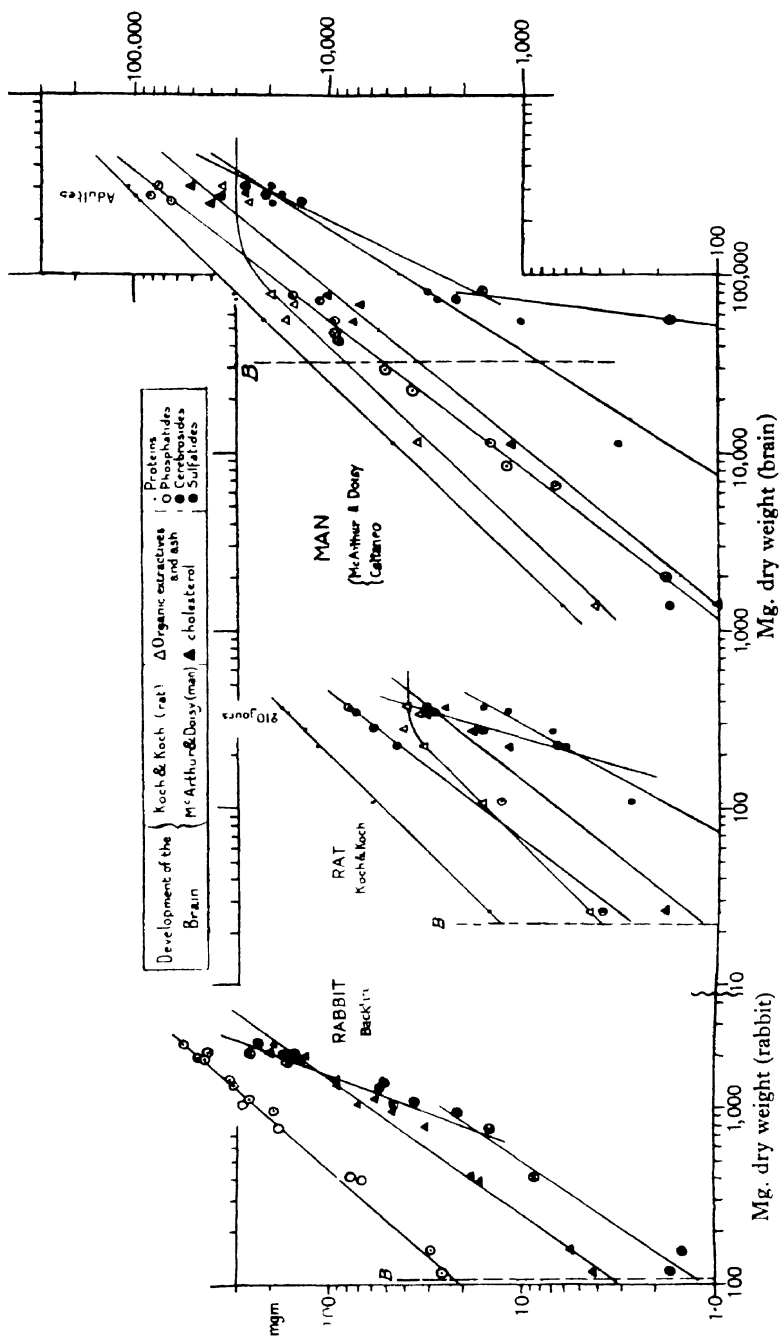


Fig. 14. Growth of the constituents of the brain of the rat (Koch and Koch, 1913), the rabbit (Backlin, 1930), and man (McArthur and Doisy, 1919; Cattaneo, 1931, 1932).

Table III. *Heterogony of the constituents of the mammalian brain.*

Fig.	Constituent	<i>k</i>			Range of <i>k</i>	Average <i>k</i>
		Rat	Man	Rabbit		
13	Related to wet weight of brain:					
	Dry substance	1.38	1.33	—	0.05	1.35
14	Related to dry weight of brain:					
	Protein	0.94	0.94	—	0.0	0.94
	Phosphatides	1.21	1.17	1.03	0.18	1.14
	Cerebrosides (first phase)	3.11	7.11	1.35	—	—
	Cerebrosides (second phase)		1.86	2.35	—	—
	Sulphatides	1.66	1.43	—	0.23	1.54
	Organic extractives and ash (until the point of completion)	0.92	0.95	—	0.03	0.94
	Cholesterol	1.15	1.12	1.28	0.16	1.18

is practically no lipid. It is as if the proteins represented the steel framework, and the lipids the concrete walls, of a modern building. There is, moreover, a certain reversibility in this succession, for May (1929, 1930) has shown that during the course of degeneration of nervous tissue (whether produced by traumatic encephalitis or by section of the sciatic nerve) there is an increase in percentage of water and nitrogen and a decrease of lipid. It is almost as if the chemical differentiation were retracing its course, but no doubt the phenomena of degeneration in nervous tissue are too complicated to admit of so simple a description. Nevertheless, the heterogony of degrowth remains an important problem, and it would be of great interest to determine whether, in an organism such as a planarian worm, where degrowth can be observed on an imposing scale, the chemical heterogony is strictly reversible. There are already grounds, drawn from observations on mammals, for supposing that it would be found to be so.

IX. EFFECT OF NUTRITIVE FACTORS ON CHEMICAL HETEROGONY.

It was remarked in connection with the heterogony of ash (Fig. 10) that the process of chemical differentiation seemed to be independent of the nutritive conditions of the animal. This phenomenon is shown again in Fig. 15, which gives Bishop's data (1929) for the lead in the chick embryo. The entry of lead into the chick follows the same course no matter what the level of lead in the unincubated egg, fixed by the diet of the laying hen, may be.

X. EFFECT OF TEMPERATURE ON CHEMICAL HETEROGONY.

Does temperature affect the heterogony of chemical substances? Can the composition of the body at a given moment be varied by changes of external temperature, *i.e.* by speeding up or slowing down, the rate at which the whole growth process takes place? At present there are insufficient data in the literature to give a satisfactory answer to this question, but a beginning has been made by

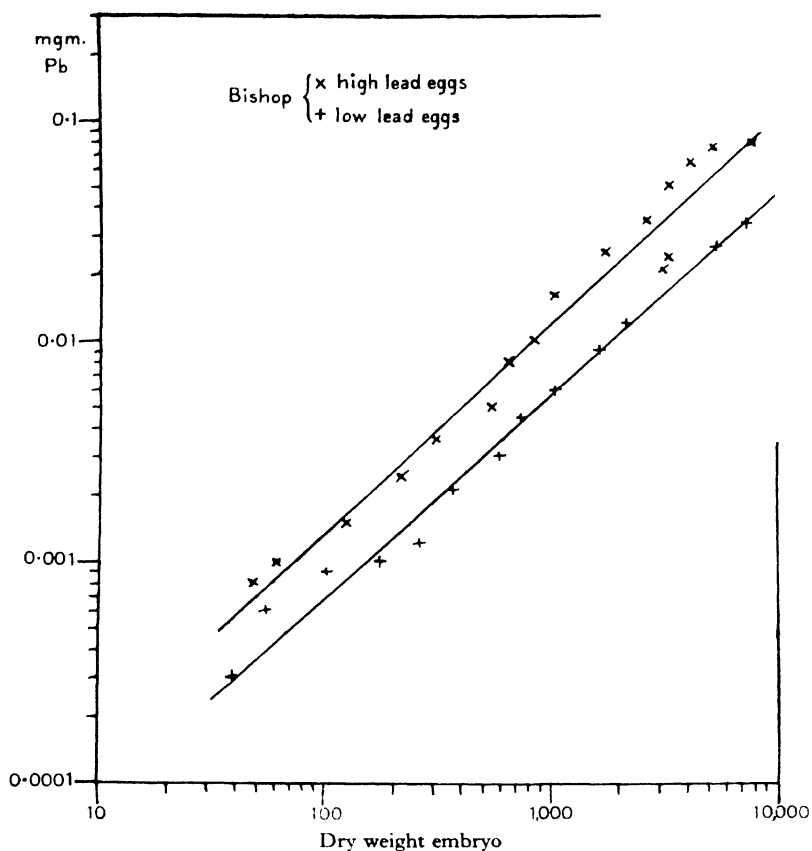


Fig. 15. Lead in the chick embryo (Bishop, 1929).

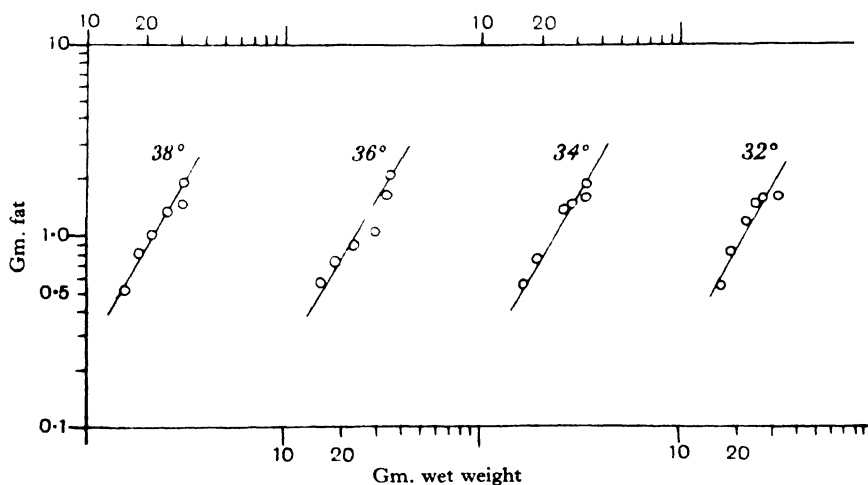


Fig. 16. Fat in the chick embryo incubated at different temperatures (Romanov and Faber, 1933).

expected merely from its size, on the contrary it follows a course inclined somewhat obliquely to the general average line. In its small stages it produces more heat than would be expected, in its late large stages less. The data of Brody and his collaborators (1932) plotted heterogonically are shown in Fig. 18, a graph which is

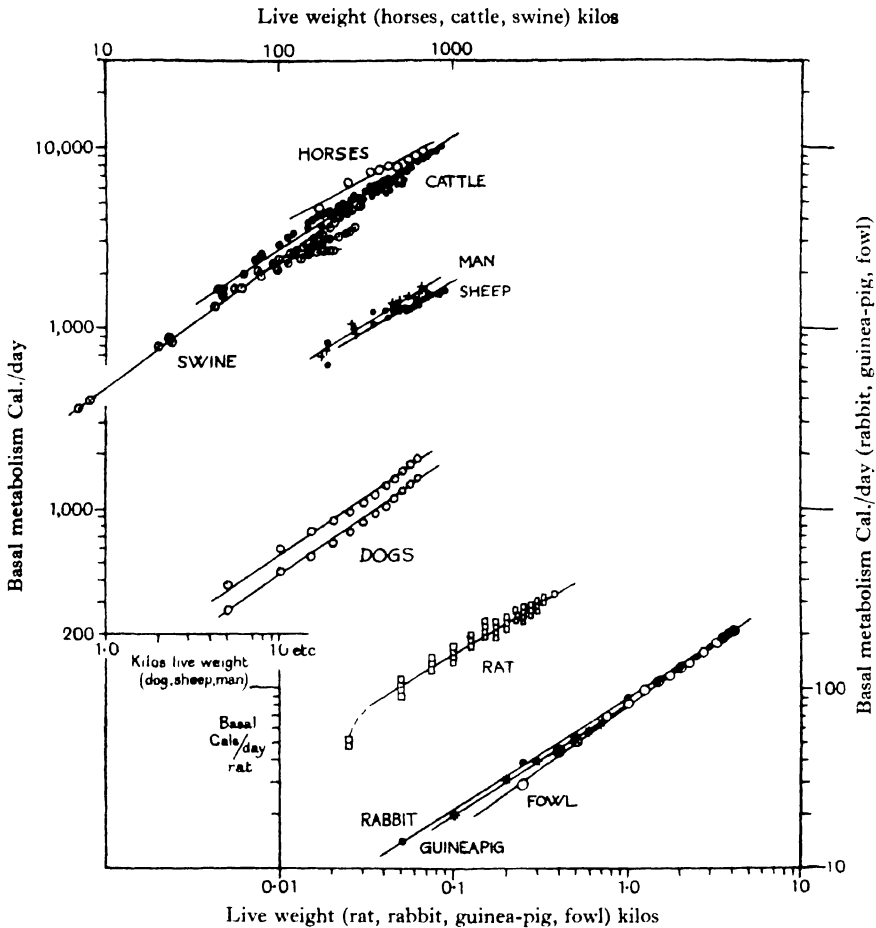


Fig. 18. Heat-production in horses, cattle, swine, humans, sheep, dogs, rats, rabbits, guinea-pigs, and fowls, from data collected by Brody *et al.* (1932).

not strictly comparable to those which have preceded it since it uses Cal./day instead of cumulative Calories. It is clear that much of the data does not fall properly upon straight lines, *e.g.* especially the swine and cattle. If, however, straight lines are roughly drawn, and their constants written down, it is found, as in Table II, that considerable variation between them exists, more than in any of the cases so far considered, but that they uniformly show an extreme negative heterogony.

XII. THE CHEMICAL GROUND-PLAN OF ANIMAL GROWTH.

Let us now consider what is the general significance of the work reviewed in this account. When we plot the magnitude of a chemical constituent of an organism against the magnitude of the organism as a whole, we ascend in this comparison to a rather high level of abstraction.

(1) We are abstracting from the morphological form.

(2) We are abstracting from the factors of nutrition.

(3) We are abstracting from the absolute values of the magnitudes. The relation of chemical entity to chemical totality may run an identical course in organisms of widely different size.

(4) We are abstracting from the time factor. At whatever speed the process of growth takes place, or whatever its position on an absolute time scale, the relation of chemical entity to chemical totality will be manifested as the same if it is the same.

What, then, is left? Nothing but a system of ratios or relations, which may possibly be the same in all animals, and which certainly seems to be the same in many animals, in a word, a chemical ground-plan of animal growth. The disturbing influence of time makes this plan difficult to see when growth is considered as a function of time, but in heterogonic plotting, the time factor is short-circuited, *i.e.* made implicit, and the plan revealed.

It may be well to enlarge upon the processes of abstraction listed above. The fact that organisms of extremely different morphological form give identical differential growth ratios for a given chemical substance might be taken to mean that genetic differences and interphyletic differences occur only at a supra-chemical level. Not "protoplasm" only, but also the changes which it undergoes in chemical constitution during the growth of the organism, would then be identical in all animals, and the genes would produce different rabbits out of the same hat, just as the sculptor produces a variety of forms out of a homogeneous substance. But it must be remembered that chemical heterogony, as so far discussed, refers to two separate levels of analysis. Out of the seventeen substances which have been discussed as chemical entities in the preceding account, seven are chemically well-defined (*e.g.* water, glycogen, lead) and ten are more properly speaking groups of substances (*e.g.* non-protein nitrogen, ash, sulphatides). The problem of how far every well-defined chemical substance in the body will be amenable to heterogonic treatment must await further work. Meanwhile, it is clear that even in the extreme case where every given group of substances had the same heterogony in different animals, there would still be a great deal of scope for genetic differences at the chemical level. "Sulphatide," for example, might show a *k* identical for supra-oesophageal ganglion and for brain, but its chemical structure (position and nature of side chains, etc.) might vary considerably as between Crustacea and mammals. One has only to think of the specificity of animal pigments or of immunologically active proteins to see that whatever else uniform heterogonic relationships mean, they do not necessarily mean that "protoplasm" is the same everywhere. The

classical work of Reichert and Brown (1909) on haemoglobins, and of Reichert (1913) on starches, need only be cited in this connection. Again, the studies on the racemisation of proteins (Dakin and Dudley, 1913; Dudley and Woodman, 1915) showed that profound interspecific differences exist in the molecular structure of different proteins. If "protein," therefore, gives us an identical k for different animals, it is much more likely to be due to some question of colloidal stability, as suggested above.

This brings up the question of how far senescence (a process obviously inclusive of that of growth) affects chemical heterogony. For the present, although an association between senescence and colloidal stability is guessed at (cf. Dhar, 1932), we cannot suppose that age as such has much to do with chemical heterogony.

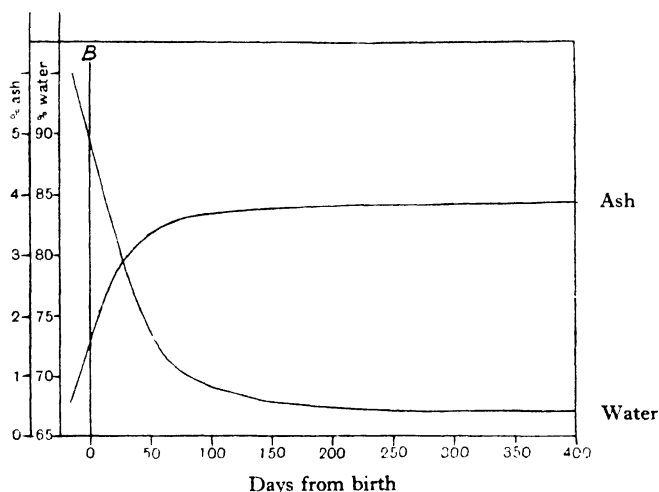


Fig. 19. Percentage of water and ash in the rat throughout the life cycle (modified from Moulton (1923); data of Hatai, Zuntz, Inaba, Chanutin, Buckner and Peter, and Sinclair).

After the cessation of growth, there is little change in chemical composition until the end of life, as is well shown in Fig. 19, modified from Moulton (1923).

As regards the validity of the conclusions drawn in this paper from similarities in k , it is clear that the main difficulty lies in knowing how close values of k must be in order to permit a conviction of identity. This is, of course, essentially a statistical problem. Unfortunately, from the ranges of k given in Tables I, II and III, it is impossible to argue far, for in each case k depends, at least partly, on the standard deviation, probable error, etc., of each of the individual sets of data on which it is founded, and in no case are the statistical qualities of the data known. Nor can they now be calculated, in view of the peculiar conditions attaching to each individual investigation and the particular chemical method employed. Since the data are so heterogeneous it is surprising that we find as many regularities as we do, and these can hardly be thought the result of chance or coincidence. What is required is a new and extensive investigation, carried out with the same chemical

methods and sampling, on a selected number of widely different organisms at all stages of the life cycle and, if possible, under constant, known, and varied, environmental conditions. The present account probably goes as far as it is possible to go by simple analysis of the published information.

It seems improbable, however, that the correspondences already noticed are illusory. In Fig. 10, for example, where the range of variation of k is rather wider than usual (0.14), it may be observed that the range for the chick embryo alone is over half the total range, *i.e.* 0.09. This may be interpreted as favourable to the view that the constants would correspond more closely than they do if the data were better.

The present work constitutes a step towards a theory of biochemical transformations. It cannot be too much emphasised that although the time factor is short-circuited in heterogonic graphs, it is not completely eliminated from the problem. On the contrary the chemical heterogonies of different animals should fuse into one common plan if they were reduced to the same time scale and the same absolute size. This has been shown by Waddington (1933) in the following way. If we have two animals p and q , and measure in each of them several chemical magnitudes M_p , M_q , N_p , N_q , etc., we find relations of the type

$$\log \frac{M_p}{\bar{M}_{p_0}} = k \log N_p \quad \dots\dots(10),$$

and
$$\log \frac{M_q}{\bar{M}_{q_0}} = k \log N_q \quad \dots\dots(11),$$

where \bar{M}_{p_0} and \bar{M}_{q_0} are specific constants, and k a general constant relating M and N for all animals. Now M and N are also functions of the time t . If we have

$$\log \frac{M_p}{\bar{M}_{p_0}} = F(t) \quad \dots\dots(12),$$

and
$$\log \frac{M_q}{\bar{M}_{q_0}} = F(t) \quad \dots\dots(13),$$

we can choose another variable ϕ such that

$$F(\phi) = F(t) \quad \dots\dots(14).$$

That is to say, by choosing a suitable unit or function for the measurement of time, we can convert the growth curve of M_p into that of M_q ; and, further, the same system of time measurement will convert all the growth curves of chemical magnitudes of animal p into those of animal q , provided only that in each case there is a linear relation between the logarithms, with the general constant k . Then we could regard the two systems of time measurement defined by t and ϕ as the relative time scales of chemical development in the two animals. But relative time scales may be derived in other ways, *e.g.* from morphological development, and from embryological determination. The morphological stage at which an organ becomes embryologically determined may vary widely in related species. As Waddington points out, it would be very interesting to know how the time scales of morpho-

logical, determinative, and chemical development, are related to one another. But this will imply the writing of a new chapter in embryology.

If, then, the unitary chemical ground-plan of animal growth exists, we must think of it as deformable in space-time. Just as d'Arcy Thompson was able (1917), by systematic deformations of Cartesian co-ordinates, to transform one morphological shape into another (*e.g.* the sun-fishes, the amphipods, mammalian skulls, etc.), so the chemical ground-plan is deformed in time and space. It can be slowed down or speeded up, and it can vary dimensionally from the extreme of the ichthyosaur to the extreme of the pocket gopher. Woodger's cones (1931) can obviously vary in absolute size and in absolute and relative length¹. But for the most part if the spatial magnitudes are reduced, the temporal magnitudes will be reduced too, and it is here that we touch upon the thought of Lambert and Teissier (1927) who have proposed as a fundamental biological law that homologies exist between animals in time as well as in space. There is an equality, they suggest, between the ratio of homologous spatial magnitudes and the ratio of homologous times, and they support their "theory of biological similarity" by the existing empirical data for cardiac frequency, metabolic rate, gestation time, longevity, etc. At one point, they closely approach the present discussion, for they suggest that "at homologous instants (in the life cycle) two homologous organs will have the same qualitative and quantitative (chemical) composition." Mouse time must bear the same, or a similar, relation to elephant time as mouse spatial magnitudes to elephant spatial magnitudes. Indeed, unless the time factor is brought into account, we may understand morphological similarity, but we can never hope to understand physiological, still less embryological similarity. Similar ideas have found expression, though not very clearly, in short notes by Carrel (1931) and du Nouy (1932), who wish to speak of "physiological time."

Lambert and Teissier, then, suggest that animals of the same general form, but of different sizes, have the same form in space-time. The investigation of chemical heterogony leads to the suggestion that animals of different form and different sizes have the same basic general chemical plan of growth, which is deformable within wide limits in space-time. The process of growth would thus proceed according to a definite plan recognisable in the constitution of the organism at any given stage of its life history. Potentiality offers to Actuality a formula in which substitution may be freely made from a wide, but not infinite, range of values.

XIII. SUMMARY.

1. In the preceding pages the application of the concept of heterogony to the chemical changes in growing Metazoa has been discussed.
2. A number of concrete examples have been brought forward which seem to indicate a uniformity of chemical heterogony in widely different organisms.

¹ The preliminary results of Moment (1933) seem to show that the water content of the organs of the rat varies solely with size if the animals are allowed to grow at widely different rates.

3. Hence it is suggested that there exists a fundamental chemical ground-plan of animal growth, capable of very varying expression in space-time.

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ESQUISSE D'UNE HISTOPHYSIOLOGIE COMPARÉE DU REIN DES VERTÉBRÉS

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(Avec Trois Figures.)

SOMMAIRE.

	PAGE
I. Introduction	110
II. Le glomus et le glomérule	114
III. Le néphrostome et le canal néphrostomial	115
IV. Le segment à bordure en brosse	116
V. Le segment à bâtonnets	121
VI. Le pouvoir athrocytaire du segment à brosse	122
VII. Le pouvoir phagocytaire du segment à brosse	126
VIII. Athrocytose et résorption de substances diffusibles	127
IX. Considérations phylogénétiques	128
X. Résumé	129
XI. Summary	130
Index bibliographique	130

I. INTRODUCTION.

DANS toute la lignée des Métazoaires, sauf chez la plupart des Trachéates, les organes présidant à la fonction excrétoire peuvent se ramener morphologiquement à un schéma d'application générale: ils se composent de tubes ou néphrons (néphridies, tubes urinaires) ouverts d'un côté dans le cœlome par un entonnoir cilié ou néphrostome, de l'autre à l'extérieur du corps. Les néphrons présentent le long de leur trajet des segments différenciés cytologiquement et physiologiquement que l'on retrouve, plus ou moins modifiés, chez tous. Chez les Invertébrés, la modification morphologique la plus importante est la perte de communication avec la cavité générale, soit que l'entonnoir cilié soit remplacé par un bouquet de cellules terminales ou solénocytes (Trématodes, certains Polychètes, etc.), soit que l'ouverture centrale du néphron soit borgne (Hirudinées), soit encore que la communication initiale de la néphridie s'établisse non plus avec la cavité générale, mais avec une poche plus ou moins réduite ayant la valeur d'un diverticule cœlomique (sacculé du Péripate et des Crustacés, cavité péricardique des Mollusques). Chez les Vertébrés, les variations dans la morphologie du néphron sont beaucoup moins grandes.

Ce qui le caractérise essentiellement, c'est l'apparition d'un glomérule capillaire; celui-ci, qui d'abord n'a que des rapports médiats avec le néphron (glomus pronéphritique des Cyclostomes et des Amphibiens), s'applique bientôt étroitement contre sa paroi, qu'il déprime, en même temps que se perd la communication avec le cœlome. Le néphron est alors en rapport étroit avec le système sanguin, et seul, l'endothélium vasculaire doublé d'une couche de péricytes, sépare le milieu intérieur,

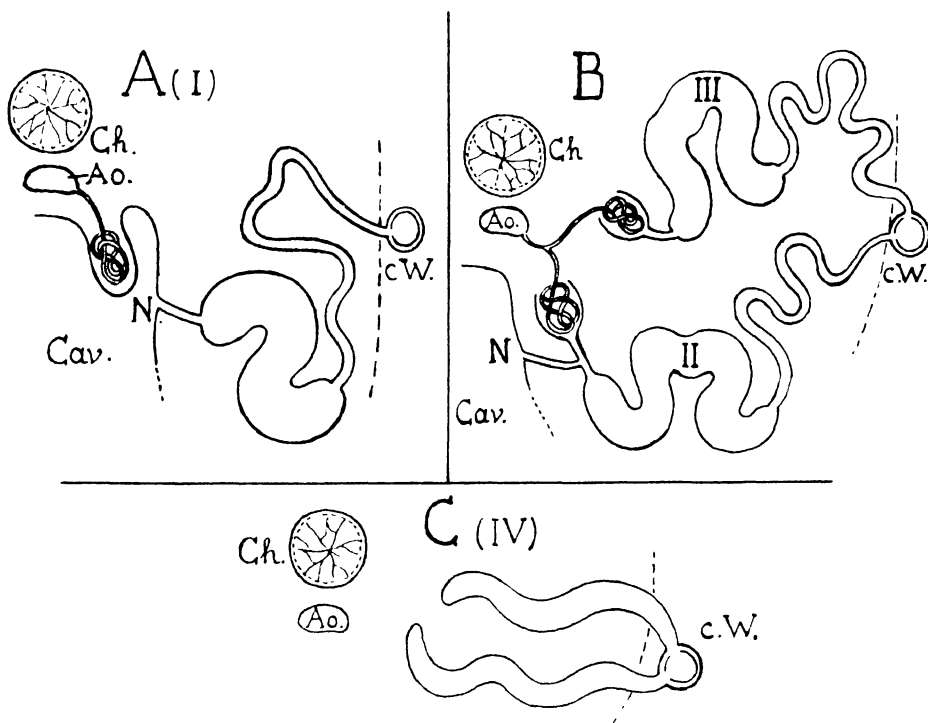


Fig. 1. Les quatre types de néphrons. En A, néphron du type I, glomus faisant saillie dans la cavité générale (Cav.), irrigué par une branche de l'aorte (Ao.). En face de lui s'ouvre le néphrostome (N.), suivi du canal néphrostomial, s'abouchant dans le segment à brosse (large); le segment à bâtonnets (étroit) lui fait suite et s'ouvre dans le canal de Wolff (c.W.). En B, sont réunis un néphron du type II et un autre du type III (disposition existant dans le mésonephros des Urodèles adultes); pas de glomus, mais des glomérules de Malpighi, irrigués par une branche de l'aorte. En C, néphrons du type IV, ni glomus, ni glomérule de Malpighi. Pas de circulation artérielle. Pas de segment à bâtonnets.

le sang, de la lumière urinaire. La dilatation initiale du néphron, qui entoure étroitement le glomérule capillaire et constitue la cavité glomérulaire, peut être d'ailleurs homologuée à un diverticule cœlomique, correspondant au saccule des Crustacés ou du Périopate.

Les divers types de néphrons rencontrés chez les Vertébrés peuvent se ramener au schéma suivant, dans lequel nous désignerons comme néphrons ouverts ceux qui ont conservé une communication directe avec la cavité cœlomique, et comme néphrons fermés ceux chez lesquels cette communication s'est perdue (Fig. 1).

Néphrons ouverts.

Type I, à glomus : pronéphros des Cyclostomes et des Amphibiens ; mésonéphros larvaire de *Discoglossus pictus*.

Type II, à glomérule¹ : mésonéphros adulte des Urodèles (*pro parte*).

Néphrons fermés.

Type III, glomérulaire : mésonéphros adulte des Sélaciens, des Téléostéens et des Anoures ; mésonéphros des Urodèles (*pro parte*) ; méso- et métanéphros des Amniotes.

Type IV, aglomérulaire : mésonéphros adulte de certains Téléostéens : Lophobranches, *Lophius*, *Opsanus*.

Au point de vue microscopique, les néphrons des types I, II et III sont caractérisés par la présence de deux segments toujours constants : un segment à brosse (voir paragraphe IV) et un segment à bâtonnets (voir paragraphe V). D'autres segments moins constants, qui présentent des variations suivant les espèces, s'intercalent entre ces deux segments principaux : collet glomérulaire, en général cilié, segment grêle cilié (Amphibiens, Reptiles) ou non (Mammifères), segments d'union et tubes collecteurs ; ceux-ci sont le siège, chez certaines espèces (Ophidiens et Lacertiliens), de variations sexuelles cycliques du plus haut intérêt biologique (Regaud et Policard, 1903 ; Cordier, 1928 ; Herlant, 1933).

Au contraire, le segment à bâtonnets manque dans les néphrons du type IV, qui ne comprennent donc qu'un segment à brosse suivi immédiatement des tubes excréteurs (Marshall et Gräfflin, 1928 ; Edwards, J. G., 1929). La présence du segment à bâtonnets semble donc liée à l'existence d'un glomérule, c'est-à-dire d'un appareil filtrant spécialisé. Le néphron le plus répandu est celui du type III ; c'est à lui que l'on songe en parlant du tube urinaire des Vertébrés ; c'est de lui que se sont occupés quasi exclusivement tous les physiologistes dans leurs recherches sur le mécanisme de la sécrétion urinaire.

Il est essentiellement caractérisé par la perte de la communication cœlomique et par la présence d'un glomérule de Malpighi. La transition entre néphron de type II et néphron de type III peut s'observer aisément chez les larves d'Amphibiens par exemple. Ainsi dans le mésonéphros larvaire de *Discoglossus pictus*, on rencontre des néphrons débutant par un glomérule de Malpighi et présentant d'autre part une communication directe avec le cœlome par l'intermédiaire d'un canal néphrostomial s'ouvrant dans le collet (type II) ; cette dernière communication se perd aux approches de la métamorphose par un mécanisme curieux que nous avons exposé ailleurs (Gérard et Cordier, 1933 *b*). Ainsi le type II devient un type III. C'est à propos du néphron de type III, le plus répandu d'ailleurs et qui est le seul type rencontré chez les Vertébrés supérieurs, que se sont posés les problèmes du mécanisme intime de la sécrétion urinaire, spécialement ceux concernant l'existence de processus de sécrétion ou de résorption dans les tubes contournés (segments à

¹ Chez les Téléostéens, le glomus pronéphritique est complètement isolé de la cavité générale, et plonge dans une chambre pronéphritique, homologue du néphrostome.

brosse). Il n'en est pas moins vrai que les mêmes problèmes se posent pour les autres types de néphrons, si longtemps négligés par les chercheurs. Il n'y a en effet pas si longtemps que les reins aglomérulaires (type IV) ont commencé à retenir l'attention des physiologistes (Verne, 1922; Marshall, 1930; Marshall et Gräfflin, 1928; Edwards et Condorelli, 1928).

Pendant de longues années, l'histophysiologie rénale a été étudiée à la lumière de deux théories déjà anciennes, qui, s'excluant l'une l'autre dans leur énoncé même, oppoisaient très nettement leurs partisans respectifs. Ce n'est que depuis peu que des tendances nouvelles se manifestent cherchant à libérer l'histophysiologie rénale des cadres rigides que lui imposaient les théories et à mettre en valeur la part de vérité contenue dans chacune d'elles. Il n'est pas téméraire d'affirmer que l'existence de ces deux formules anciennes, la théorie de Bowman et la théorie de Ludwig, établissant pendant longtemps entre les deux camps adverses un mur infranchissable, a été un obstacle à la claire compréhension des mécanismes biologiques se déroulant dans l'organe rénal.

Théorie de la sécrétion (Bowman-Heidenhain) et théorie de la résorption (Ludwig-Cushny) sont suffisamment classiques pour en rendre superflu un exposé détaillé. Résumons-les brièvement: toutes deux s'accordent à reconnaître au glomérule une fonction de filtration s'exerçant aux dépens du sérum sanguin, dont les éléments diffusibles traversent le filtre. Au niveau des segments à brosse, la théorie de Bowman place un processus de sécrétion ou plutôt d'excrétion, par lequel s'éliminent directement, dans le liquide tubulaire, des catabolites puisés dans le milieu intérieur par la base des cellules. La théorie de Ludwig nie cette sécrétion et admet au contraire que le segment à brosse résorbe certaines substances présentes dans le liquide tubulaire pour les restituer à l'organisme par sa membrane basale. En même temps une résorption d'eau transforme le liquide intracellulaire en urine définitive. En d'autres mots, Bowman suppose l'enrichissement en substances dissoutes du filtrat glomérulaire pendant son passage dans les tubes, tandis que Ludwig admet au contraire son appauvrissement en eau et en substances dissoutes. Dans l'esprit des théoriciens, sécrétion et résorption doivent s'exclure. D'innombrables travaux se sont attachés à établir le bien fondé de l'une ou de l'autre des théories. D'une façon générale, les auteurs qui arrivaient à fournir un argument en faveur de l'une se sont toujours crus obligés de nier toute valeur à l'autre. Les efforts pour concilier les deux opinions sont de date relativement récente.

L'étude de l'histophysiologie comparée des Vertébrés permet d'aborder avec fruit le problème de la fonction rénale. L'existence des diverses dispositions morphologiques décrites plus haut nous met à même, en effet, par un choix judicieux du matériel, de dissocier en quelque sorte les diverses parties constituant le néphron et d'en examiner isolément les potentialités physiologiques. Ainsi s'obtiennent des résultats que n'aurait pu fournir l'étude exclusive de néphrons de type III.

Quatre segments essentiels sont à examiner au point de vue de l'histophysiologie du néphron: le glomus ou le glomérule, le néphrostome et son canal néphrostomial, le segment à brosse (tube contourné) et le segment à bâtonnets. Nous les envi-

sagerons l'un après l'autre en exposant brièvement quelles propriétés physiologiques essentielles l'on peut reconnaître à chacun d'eux. Nous terminerons par quelques considérations sur une potentialité importante que nous verrons se dessiner au cours de cet exposé : le pouvoir athrophagocytaire du segment à brosse.

II. LE GLOMUS ET LE GLOMÉRULE.

Le processus de filtration qui siège au niveau du glomérule de Malpighi n'est actuellement mis en doute par personne. Le rôle du glomérule comme piston propulseur (Lamy et Mayer, 1906) est actuellement entièrement abandonné. Depuis les belles recherches de l'école de Richards, on admet que tous les constituants diffusibles du sérum sanguin passent à travers les endothéliums glomérulaires. Wearn et Richards (1925), prélevant au moyen d'une micropipette le liquide filtrant dans la cavité glomérulaire du rein de la Grenouille, et le soumettant à des tests chimiques, y ont trouvé de l'urée, des chlorures, et aussi du glucose. Les colorants diffusibles (indigocarmin, rouge phénol), injectés dans les veines, se retrouvent, mais très dilués, dans le filtrat glomérulaire. Il en est de même pour l'acide urique injecté ainsi que nous l'avons montré par d'autres méthodes (Gérard et Cordier, 1932 *a*).

Les très nombreuses recherches faites au moyen de l'emploi de colorants colloïdaux acides, type trypanblau (*v.* notamment von Moellendorff, 1915), ainsi que d'autres corps colloïdes, ont mis en évidence que les substances colloïdes de haute dispersion (trypanblau, carminates de lithium ou d'ammoniaque, encre de Chine RAL, saccharate de fer) peuvent filtrer par le glomérule, alors que des colloïdes moins disperses sont régulièrement arrêtés (encre de Chine Pelikan 541, bleu de Prusse soluble, oxyde de thorium colloïdal, solution colloïdale de cholestérol (Gérard et Cordier, 1933 *c*)) et ne peuvent franchir la barrière glomérulaire.

Les albumines étrangères sont également éliminées par le glomérule (Nussbaum, 1877-8) et entraînent fréquemment au niveau des endothéliums glomérulaires des lésions se traduisant par une augmentation de la perméabilité à la faveur de laquelle peuvent passer dans l'urine des substances habituellement retenues, cholestérol, albumines du sérum (Asai, 1928; Gérard et Cordier, 1923 *c*). Récemment Bayliss, Kerridge et Russell (1933) ont montré que chez le Mammifère, le glomérule est perméable à des protides dont le poids moléculaire ne dépasse pas 70 000 (gélatine, ovalbumine, hémoglobine).

Cette filtration d'albumines par le glomérule est bien démontrée depuis que l'on a constaté (Nussbaum, 1877-8; Gérard et Cordier, 1933 *c*) que la suppression de la fonction glomérulaire empêche leur passage dans l'urine. D'autre part, l'on sait que des facteurs amenant habituellement de l'albuminurie (asphyxie, injections d'ovalbumine, de sublimé) sont impuissants à la provoquer chez des animaux à reins aglomérulaires (Bieter, 1931). Il n'est pas douteux que le processus de filtration mis en évidence au niveau du glomérule est aussi l'apanage du glomus pronéphritique. Le glomus pronéphritique des Téléostéens laisse filtrer, dès le moment où il est constitué, le trypanblau, comme le glomérule lui-même (Cordier, 1932).

Peut-être sa perméabilité est-elle moins grande, car il arrête habituellement des substances comme les carminates, et le saccharate de fer, que le glomérule laisse passer. Chez les larves d'Amphibiens, le liquide filtrant par le glomus est aspiré par le battement très actif des néphrostomes ciliés, situés immédiatement en regard de lui. Ainsi se crée, dans le tube urinaire pronéphritique, le courant liquide indispensable à l'établissement de la fonction excrétoire. Nussbaum a montré (1886) que les canaux de Wolff, chez l'Amphibien et le Téléostéen, renferment très tôt des cristaux de nature uratique qui se dissolvent dès que le glomus est constitué.

Nous sommes donc en droit de croire, que, dans les néphrons à glomus ou à glomérule, qu'ils soient ouverts ou fermés (types I, II, III), c'est essentiellement le floccule capillaire (c'est-à-dire le rete mirabile du glomus ou du glomérule) qui fournit l'apport liquide de l'urine. Le canal néphrostomial, dans les néphrons ouverts, ne pourrait le faire, le cœlome ne contenant évidemment pas assez de liquide pour fournir un flux suffisant. Pour ce qui concerne le néphron de type IV, il n'en va évidemment pas de même; ainsi, le rein aglomérulaire de *Lophius* fournit des quantités d'urine très considérables, dont l'eau résulte évidemment d'un processus autre que la filtration (Edwards et Condorelli, 1928).

Un autre point intéressant à signaler au point de vue de l'histophysiologie comparée, c'est le rapport qui semble exister entre le nombre et le développement des glomérules d'une part et l'habitat d'autre part. Les Poissons de mer, vivant dans un milieu hypertonique, réduisent leur perte en eau par une réduction du volume des glomérules (Nash, 1931; Marshall et Smith, 1930), alors que chez les Téléostéens d'eau douce les glomérules sont beaucoup plus grands. Chez les Reptiles, Cordier (1928) a montré, par des reconstructions, que la richesse du réseau filtrant glomérulaire est en rapport étroit avec la densité de l'urine; dans la série Chéloniens, Ophidiens, Lézards, la surface filtrante va en diminuant; parallèlement à ce fait, on constate que les Tortues ont encore une urine liquide, alors qu'elle est solide dans les deux autres groupes de Reptiles.

III. LE NÉPHROSTOME ET LE CANAL NÉPHROSTOMIAL.

Néphrostome et canal néphrostomial ne semblent avoir chez les Vertébrés où ils existent (type I et II) qu'une importance réduite.

Ils n'existent en effet jamais seuls, et nous pouvons les considérer plutôt comme des restes de la néphridie primitive. Leur tendance à la disparition s'affirme dans les groupes inférieurs: les Téléostéens n'en ont plus; chez les Amphibiens, seules les espèces les moins évoluées en possèdent. Il est vrai que le rein adulte des Anoures montre encore des néphrostomes, mais ceux-ci s'ouvrent dans les veines, et n'interviennent donc plus du tout dans la physiologie du néphron. Dans le pronéphros des Amphibiens, le rôle du néphrostome semble être purement mécanique, les longs cils attirant vers le néphron le liquide filtrant par le glomus. Dans le mésonephros, chez les larves des Anoures ou chez les Urodèles adultes, cette action d'aspiration peut s'exercer tout au plus sur la faible quantité de liquide contenue dans le cœlome.

Dans le mésonéphros des Anoures adultes, il est intéressant d'observer que les battements des cils néphrostomiaux suffisent à créer une pression équilibrant parfaitement celle existant dans les capillaires veineux où s'ouvre le canal néphrostomial; on n'observe en effet jamais d'écoulement de sang vers le cœlome. Si au contraire le mouvement des cils se ralentit, ce qui se produit au moment de la fixation des reins aux fins de leur étude histologique, il est fréquent d'assister à une légère hémorragie, les érythrocytes traversant à rebours le canal néphrostomial pour tomber dans la cavité cœlomique.

Le canal néphrostomial du mésonéphros larvaire des Anoures possède cependant un pouvoir athrocytaire (voir paragraphe VI) capable de s'exercer envers les colloïdes injectés dans le cœlome, mais ce pouvoir ne se manifeste qu'après que le néphrostome a perdu toute communication avec le néphron et dès qu'il s'est mis en communication directe avec le sang (Gérard et Cordier, 1933 a). Avant ce moment, c'est-à-dire pendant le laps de temps où il s'ouvre dans le collet glomérulaire des néphrons, cette propriété d'athrocytose n'existe pas.

IV. LE SEGMENT À BORDURE EN BROSSE.

(*Tube contourné.*)

Ce segment se retrouve chez la plupart des Vertébrés avec les mêmes caractéristiques cytologiques, à savoir: (1) une bordure en brosse, plus ou moins nettement différenciée suivant les états fonctionnels, mais toujours présente; elle est formée d'une grande quantité de fins prolongements parallèles, situés au pôle apical, semblables à des cils raides, mais non doués de mouvement; (2) des granulations apicales, d'abondance et d'aspect variables. Ces enclaves, qui sont constantes chez les Vertébrés à sang froid, ont souvent une teinte jaunâtre et sont probablement de nature lipopigmentaire. Chez les Vertébrés à sang chaud, elles ne se rencontrent guère, sauf cependant chez les Mammifères en hibernation. Elles sont donc très probablement en rapport avec un métabolisme plus lent, que celui-ci soit normal comme chez les animaux poikilothermes, ou occasionnel comme chez les Mammifères hibernants.

C'est au niveau du segment à brosse que les physiologistes situent les processus les plus importants contribuant à la préparation de l'urine: sécrétion pour les uns, résorption pour les autres.

Il serait évidemment impossible d'exposer ici, même d'une façon très brève, les innombrables expériences invoquées en faveur de l'un ou de l'autre processus. De l'ensemble de ces recherches, on est cependant autorisé à tirer quelques conclusions fermes.

(a) *Le processus de résorption.*

L'existence d'un processus de résorption, s'exerçant envers certaines substances, normalement présentes ou introduites expérimentalement, est certaine. Wearn et Richards (1925) ne retrouvent pas dans l'urine vésiculaire le glucose dont ils constatent la présence dans le liquide glomérulaire. White et Schmidt (1926),

ponctionnant chez *Necturus*, en même temps que la capsule glomérulaire, l'extrémité distale du segment à brosse, montrent que le glucose filtrant au glomérule est résorbé pendant son parcours dans le segment à brosse, à la fin duquel on ne le retrouve plus. Au point de vue histophysiologique, divers arguments ont été fournis en faveur de l'existence d'un processus de résorption. Les recherches de von Moellendorff (1915) chez la Souris et la Grenouille ont établi que les colorants colloïdaux acides s'accumulent dans le segment à brosse suivant une règle toujours constante: accumulation maximale dans les parties initiales et décroissance progressive vers les parties distales. Cette accumulation décroissante se retrouve chez tous les Vertébrés. Cordier l'a signalée chez les Reptiles (1928) et nous-mêmes l'avons pu retrouver chez les Poissons et les Oiseaux. C'est là une règle absolument générale et nous aurons à y revenir. Cette répartition a été interprétée comme résultant d'une résorption, au début du tube, du colorant filtrant au glomérule, la quantité offerte à la résorption étant évidemment la plus grande à ce niveau.

De Haan et Bakker (1923) ont montré également que l'apparition des colorants colloïdaux acides (trypanblau) dans les cellules rénales était conditionnée par l'existence d'un glomérule complètement différencié. Chez le Chat nouveau-né, les tubes urinaires en rapport avec des pseudoglomérules ne contiennent jamais d'inclusions colorées, parce que le colorant ne peut leur arriver. Von Moellendorff (1919) n'a pu constater d'accumulation de colorants acides dans les parties du mésonéphros larvaire des Anoures où le glomérule de Malpighi était insuffisamment différencié; le colorant apparaît dès que le glomérule devient fonctionnel. A ce dernier argument, on peut toujours objecter qu'avant la différenciation des glomérules, les tubes eux-mêmes sont embryonnaires, non fonctionnels, et par conséquent incapables de "sécréter" les colorants acides injectés.

Si nous nous adressons à des tissus adultes, nous voyons cependant que les colorants colloïdaux acides n'apparaissent jamais dans les cellules des segments à brosse, du moment que ceux-ci ne sont pas en rapport avec un glomérule (néphrons du type IV).

La preuve formelle du passage glomérulaire des colorants acides et de leur résorption par les segments à brosse est fournie par l'étude du comportement du rein d'Anoure après ligature des artères. Cette intervention supprime en effet la fonction glomérulaire et laisse persister uniquement la fonction tubulaire. Nussbaum l'a utilisée, il y a longtemps (1877-8), dans des recherches devenues classiques.

Nous avons employé cette même méthode chez le Crapaud (1932 a); en ligaturant les artères irriguant une moitié du rein, on supprime dans cette zone la fonction glomérulaire, tandis que l'autre moitié reste normalement vascularisée et sert de témoin. Chez des Crapauds ainsi opérés, l'injection de colorants colloïdaux acides (trypanblau, carminates, colloïdes organométalliques très disperses comme le saccharate de fer, le solganal, etc.) est suivie de l'apparition de ces substances dans les cellules des segments à brosse correspondant à des glomérules normaux tandis que ceux dont le glomérule est fonctionnellement supprimé n'en contiennent jamais (Fig. 2 A et A'). Comme nous avons pu montrer d'autre part que les épithéliums rénaux, après ligature artérielle, conservent leur intégrité morpholo-

gique et physiologique, ces expériences prouvent que les colloïdes utilisés passent par le glomérule et sont résorbés dans le tube contourné.

Quoique, à notre avis, le comportement des reins à artères ligaturées apporte une preuve formelle de l'existence de processus de résorption, certains pourraient

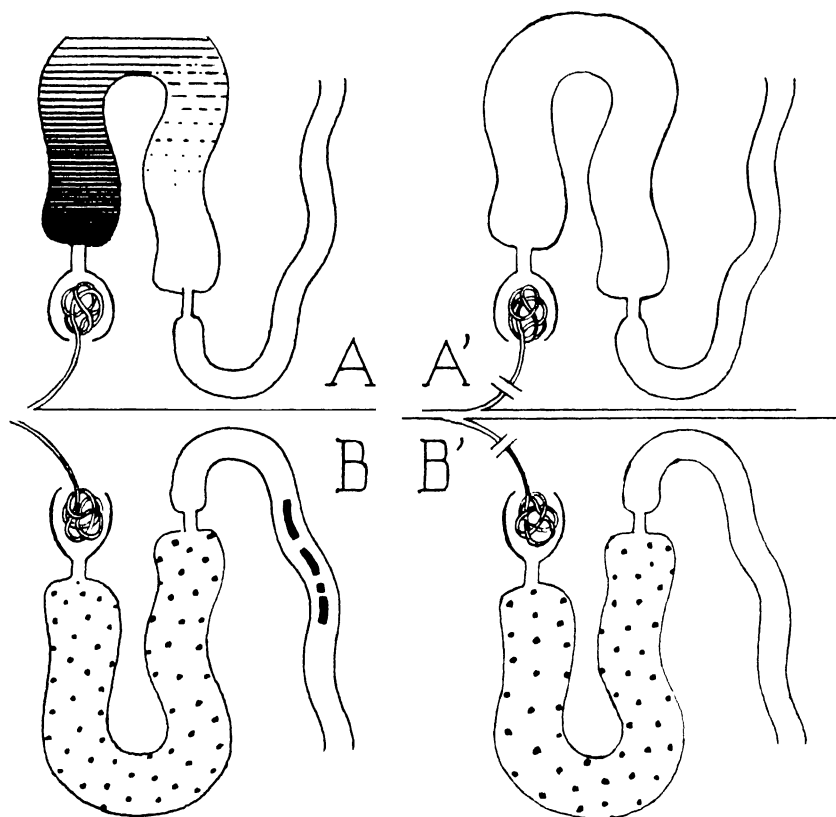


Fig. 2. Résultats expérimentaux obtenus sur des reins chez lesquels la circulation glomérulaire a été supprimée. En A, partie normale; athrocytose de colloïdes très dispersés dans le segment à brosse suivant la règle de von Moellendorff. En A', partie opérée; pas d'athrocytose dans le segment à brosse. En B, partie normale; images de sécrétion de l'acide urique. Petits cristaux uriques dans les cellules du segment à brosse. Gros amas cristallins dans la lumière du segment à bâtonnets. En B', partie opérée; cristaux d'acide urique dans les cellules du segment à brosse. Pas de masses cristallines dans la lumière du segment à bâtonnets.

cependant nous objecter que l'intervention opératoire et la suppression de la circulation artérielle ont aboli certaines potentialités excrétrices des tubes, et que l'absence des substances injectées dans les tubes contournés privés de glomérule fonctionnel pourrait peut-être s'expliquer par une déficience des propriétés sécrétoires normales. Pour rencontrer cette objection, nous possédons les résultats obtenus par l'injection de colloïdes de dispersion faible ou moyenne. Ces sub-

stances, nous le savons, ne peuvent passer par le filtre glomérulaire et il n'est pas étonnant qu'on ne les retrouve pas dans le segment à brosse, après injection. Cependant leur emploi peut donner des résultats extrêmement instructifs à condition de choisir judicieusement le type de néphron sur lequel on veut expérimenter. Les reins à néphrons ouverts, de type I ou II, vont nous fournir un matériel d'étude particulièrement intéressant et ce d'autant plus que cette fois nous emploierons des animaux parfaitement normaux. L'existence d'une communication directe entre néphron et cœlome permet, dans les néphrons de type I ou II, de porter directement au contact des cellules rénales les colloïdes faiblement ou moyennement dispersés, ce qui est impossible dans les néphrons du type III. Il suffit de pratiquer une injection dans la cavité cœlomique pour voir le liquide aspiré par les néphrostomes pénétrer dans les néphrons.

Notre élève P. Lambert (1932) a utilisé le rein de la Salamandre où se rencontrent des néphrons de type II et des néphrons de type III. Injectant dans le cœlome des colloïdes peu dispersés, bleu de Prusse soluble, encre de Chine Pelikan 541, il a pu observer que ces substances s'accumulent dans les tubes contournés des néphrons ouverts de type II, tandis que les néphrons fermés de type III n'en renferment jamais. Une sécrétion directe des substances injectées, par les segments à brosse, est absolument exclue, puisque néphrons ouverts et fermés sont placés exactement dans les mêmes conditions et que seuls les néphrons ouverts contiennent des enclaves colorées. Une seule explication des faits observés est possible: c'est la pénétration directe des colloïdes par les néphrostomes et leur résorption dans les segments à brosse des néphrons ouverts. Nous-mêmes (1933 *a*) avons pu observer des phénomènes absolument identiques chez les têtards d'Anoures. Le pronéphros du têtard, qui est formé de néphrons de type I résorbe tous les colloïdes introduits dans la cavité générale, quelle que soit leur dispersion. Quant au mésonéphros, il se comporte comme un mésonéphros d'adulte chez *Rana* et *Bufo*, tandis que chez la larve de *Discoglossus pictus* nous avons pu constater la persistance de néphrons ouverts, jusqu'aux approches de la métamorphose. Chez cette dernière espèce, l'injection de colloïdes faiblement dispersés (bleu de Prusse soluble, encre Pelikan 541) dans la cavité cœlomique est suivie de l'apparition et de l'accumulation intracellulaires de ces substances dans les segments à brosse des néphrons encore ouverts, tandis que dans les néphrons qui se sont déjà fermés par suite de la perte de leur communication péritonéale, les enclaves colorées n'apparaissent jamais.

Pour nous résumer, nous dirons que l'existence d'un pouvoir de résorption des segments à brosse, affirmée par les physiologistes, et en faveur de laquelle les expériences de l'école de Richards apportent de si puissants arguments, trouve dans l'observation histophysiologique une démonstration éclatante. Mais alors que les observations des physiologistes admettent une résorption de substances diffusibles, qui sont restituées au milieu intérieur, les preuves histophysiologiques de la résorption sont basées uniquement sur l'emploi de substances colloïdes. Or, ces substances, après résorption, sont floculées et accumulées, par un phénomène particulier, connu des auteurs allemands sous le nom de "Speicherung" et que nous avons désigné par le terme d'"athrocytose" (Gérard, 1933; Gérard et Cordier,

1933 a). Ce phénomène mérite une attention toute spéciale; nous nous en occuperons plus loin pour nous efforcer de le comprendre et d'en interpréter la signification.

(b) *Le processus de sécrétion.*

L'existence de processus de sécrétion, c'est-à-dire de passage direct de substances à travers les cellules du segment à brosse suivant le sens base-apex, a été entièrement niée par les partisans de la théorie de Ludwig-Cushny. Ce n'est que dans ces dernières années que de nombreuses voix se sont élevées pour rendre à l'activité excrétrice des cellules rénales la place qui lui revient dans la fonction urinaire. La réalité des processus de sécrétion ne fait pas de doute pour ce qui concerne les néphrons du type IV. L'on sait que les Poissons aglomérulaires sont capables de fournir une urine abondante (*Lophius*), et d'éliminer les colorants acides diffusibles (rouge phénol) injectés dans l'organisme (Edwards et Condorelli, 1928).

Il serait bien étonnant, alors que nous voyons partout une structure si uniforme du néphron dans la série des Vertébrés, que les néphrons glomérulaires, qu'ils soient ouverts ou fermés, présentassent une différence si essentielle avec les néphrons aglomérulaires, et que ceux-ci ne pouvant que sécréter, les autres ne puissent plus que résorber. Il faut cependant donner des preuves plus palpables de la sécrétion. Ces preuves avaient déjà été fournies par les recherches de Nussbaum, reprises récemment par Bensley et Steen (1928). Ces auteurs, ligaturant les artères rénales, chez *Rana*, voyaient que malgré l'absence de glomérule fonctionnel, les segments à brosse éliminaient dans leur lumière, et ce à une concentration considérable, l'indigocarmin et le rouge phénol injectés dans la circulation. Récemment, Höber et Mirowsky (1932) ont montré, chez *Rana*, que les colorants acides diffusibles non liposolubles passent par les segments à brosse des reins de Grenouille à artères ligaturées. Les recherches de ces auteurs ont mis en évidence un fait extrêmement intéressant; c'est que parmi les multiples colorants diffusibles utilisés, certains (rouge phénol) s'éliminent encore par le rein, au niveau du segment à brosse, lorsque l'on perfuse l'organe avec du Ringer, tandis que d'autres (cyanol, bleu patenté V) ne s'éliminent par ces mêmes segments que si ceux-ci se trouvent en contact du sang de l'animal. Il n'est même pas absolument nécessaire que ce sang soit circulant, car des reins excisés, placés immédiatement dans une solution diluée de cyanol, montrent après quelques minutes l'apparition de la couleur dans les lumières tubulaires et ce à une concentration de 5 à 10 fois plus forte que dans le sang.

L'élimination directe des colorants diffusibles par les tubes contournés ne fait donc pas de doute; et il ne s'agit pas là d'une simple diffusion: la substance injectée est excrétée à une forte concentration, ce qui implique évidemment un travail cellulaire. Les résultats de Höber sont encore intéressants parce qu'ils démontrent avec quelle prudence il faut accepter les conclusions obtenues par de nombreux physiologistes qui ont étudié la fonction rénale de la Grenouille sur des animaux perfusés par des liquides artificiels, genre Ringer. Nos propres recherches sur le Crapaud à

artères rénales ligaturées ont mis en évidence également le pouvoir sécréteur des tubes contournés et ce non pas envers des substances colorantes, mais envers des substances de déchet normales, notamment l'acide urique. Nous avons pu montrer que l'acide urique injecté se retrouve encore dans les tubes contournés privés de glomérule fonctionnel, mais que, le flux liquide étant supprimé, cette substance stagne sur place et n'atteint pas les segments distaux du néphron (Fig. 2 B et B'). Lueken (1932) arrive aux mêmes conclusions par des méthodes différentes. Enfin, remarquons que l'emploi de colorants vitaux basiques, rouge neutre ou bleu de méthylène, est entièrement à rejeter dans les études sur la physiologie de l'excrétion. Ces colorants pénètrent trop facilement dans les cellules, non seulement dans les épithéliums rénaux, mais encore dans une foule d'autres éléments de l'organisme; les images d'accumulation granulaire qu'ils fournissent dans les segments à brosse ne témoignent nullement d'une excrétion spécifique des colorants à ce niveau, mais indiquent simplement un processus de coloration vitale analogue à celui qui s'observe dans une foule de cellules non émonctoires.

V. LE SEGMENT À BÂTONNETS.

(Tube IV', distal convoluted tubule.)

Comme son nom l'indique, ce segment est caractérisé cytologiquement par la présence d'une striation cytoplasmique, due à la présence de bâtonnets, disposés parallèlement au grand axe des cellules, aisés à fixer et à colorer et désignés fréquemment sous le nom de bâtonnets d'Heidenhain. Ce segment ne se retrouve que dans les néphrons des types I, II et III; les néphrons aglomérulaires n'en renferment pas. Sa présence paraît donc liée à l'existence d'un appareil filtrant; ce fait morphologique semble déjà indiquer que ce segment a comme rôle de résorber de l'eau pendant le passage de l'urine à son niveau.

Le pouvoir de résorption d'eau du segment à bâtonnets—ou plutôt son pouvoir concentrateur—a été observé déjà par Nussbaum (1886) chez le Triton, à la suite d'injections d'indigocarmin. Bensley et Steen (1928) ont nettement observé par le même moyen que le colorant éliminé au niveau des segments à brosse se concentre fortement dans les segments à bâtonnets. Dans les reins à glomérules rendus non fonctionnels (par ligature artérielle), la résorption d'eau, dans les segments à bâtonnets, peut produire la précipitation et la cristallisation du colorant dans les lumières. Nous-mêmes (1932 a) avons pu observer le rôle concentrateur de ce segment chez le Crapaud; à la suite d'injections d'acide urique, l'on voit en effet se déposer, dans la lumière de ce segment, de gros amas cristallins, brillamment illuminés entre nicols croisés, indiquant une grande concentration en acide urique du liquide tubulaire (Fig. 2 B). D'autre part, l'étude de l'élimination des sels d'urane chez le Crapaud nous a conduits aux mêmes résultats; le nitrate d'urane qui filtre, très dilué, au glomérule, ne subit guère de concentration pendant son passage dans le segment à brosse, qu'il ne lèse donc pas; dès son arrivée dans le segment à bâtonnets, la résorption d'eau amène le nitrate d'urane à une concentra-

tion toxique, traduite par de fortes lésions exsudatives et desquamatives au niveau des épithéliums.

Le segment à bâtonnets est très développé chez les Mammifères; c'est en effet chez ces animaux que l'urine est la plus concentrée. Ce segment est représenté chez eux par la branche ascendante de l'anso de Henle et par le segment intermédiaire de Schweigger-Seidel.

VI. LE POUVOIR ATHROCYTAIRE DU SEGMENT À BROSSSE¹.

Le phénomène d'athrocytose ("Speicherung") est bien connu et a été très abondamment mis en évidence au niveau des éléments réunis sous la dénomination générale de tissu réticulo-endothélial². Il consiste essentiellement en une absorption de substances colloïdales, généralement électro-négatives, et leur accumulation au sein du cytoplasme, soit sous forme de granules de nouvelle formation, soit dans des vacuoles ou sur des grains préformés. Enfin, le phénomène se caractérise encore par une rétention très énergique des substances absorbées que les cellules ne lâchent que très progressivement et très lentement.

Le phénomène d'athrocytose se met en évidence au niveau du tube contourné de tous les Vertébrés par l'emploi de colloïdes très dispersés. C'est le degré de dispersion qui est le facteur essentiel réglant l'apparition du phénomène dans la cellule rénale; en effet, tout ce que nous avons exposé plus haut montre très nettement que seul le pôle apical des cellules du segment à brosse peut résorber des colloïdes et que leur base leur est absolument imperméable. Comme les auteurs se sont presque exclusivement adressés à des néphrons de type III, ils n'ont pu observer que la fixation d'une très petite catégorie de colloïdes; ceux dont les particules suffisamment fines leur permettent de traverser le filtre glomérulaire. Nous avons ainsi pu montrer que (Gérard et Cordier, 1932 *b*), en utilisant diverses marques d'encre de Chine du commerce, seule la RAL présentait les conditions requises pour filtrer par le glomérule; on la retrouve ensuite résorbée et accumulée dans les cellules des segments à brosse. Le pouvoir de résorption et d'athrocytose des cellules rénales ne se borne cependant pas à l'absorption et l'accumulation des colloïdes de haute dispersion. Des recherches d'histophysiologie comparée nous ont montré que le pouvoir athrocytaire du segment à brosse ne le cède en rien à celui des histiocytes du tissu réticulo-endothélial. Pour le démontrer, on ne peut évidemment s'adresser à des néphrons de type III, qui ne permettent l'arrivée dans la lumière des tubes que des seuls colloïdes filtrant au glomérule. Nous avons

¹ Nous préférons le terme d'*athrocytose* à celui de Chromopexie proposé par Volkonsky (1933). Il présente tout d'abord l'avantage de dériver d'un terme bien connu, *athrocyte*, proposé par Burian. Le phénomène qu'il désigne est d'ordre beaucoup plus général; il s'exerce en effet sur toute substance en solution colloïdale, quelle que soit sa composition chimique. La chromopexie est un cas particulier de l'athrocytose.

² On réunit sous la dénomination de "tissu réticulo-endothélial" un ensemble de cellules jouissant toutes de la propriété d'absorber et de fixer sous forme granulaire les colloïdes électro-négatifs injectés dans l'organisme (trypanblau, carmin, colloïdes organo-métalliques) ainsi que certaines substances endogènes (pigments d'origine hématique). Ces cellules sont en outre phagocytaires. A ce système appartiennent notamment les cellules réticulaires des organes hémopoïétiques, les histiocytes du tissu conjonctif, les cellules de Kupffer du foie, les endothéliums des sinus veineux de la rate, etc.

utilisé dans ce but des néphrons de types I et II, c'est-à-dire ceux qui, grâce à l'existence d'un canal néphrostomial, peuvent recevoir directement des substances introduites dans la cavité cœlomique sans passer par le filtre glomérulaire. Notre élève Lambert (1932) a étudié, au point de vue qui nous occupe, les néphrons mésonéphritiques ouverts (type II) de la Salamandre adulte; nous-mêmes (1933 a) avons utilisé le pronéphros du têtard d'Anoure (type I) ainsi que les néphrons ouverts du mésonéphros larvaire de *Discoglossus pictus* (type II). Les injections cœlomiques de colloïdes de dispersion moyenne (bleu de Prusse soluble, oxyde de thorium colloïdal (thorotrast)) ainsi que de colloïdes de très faible dispersion dont les particules sont à la limite de la visibilité (encre de Chine Pelikan 541) ont été suivies d'une athrocytose régulière et constante de ces substances dans les segments à brosse.

L'emploi de colloïdes de dispersion diverse nous a permis d'observer très nettement une propriété très particulière du néphron, propriété déjà soupçonnée par von Moellendorff (1915) et dont nous-mêmes avons pu constater une manifestation à la suite d'injection de tellure métallique chez le Crapaud (Gérard et Cordier, 1932 a). Voici de quoi il s'agit: les colloïdes très disperses pénétrant dans le néphron après filtration glomérulaire sont résorbés et accumulés dans les tubes contournés suivant la règle de von Moellendorff: l'accumulation est maximale au début du tube et décroît régulièrement à partir de ce niveau. Von Moellendorff avait pu cependant observer que le bleu pyrrhol, moins dispersé, faisait exception et que son maximum d'athrocytose se situait vers le milieu du tube. L'auteur en avait déduit qu'il existait le long du tube contourné une augmentation régulière de la perméabilité qui, trop faible au début pour permettre l'entrée du bleu pyrrhol, provoquait une accumulation maximale de ce colorant plus loin, là où la perméabilité était devenue suffisante; nous-mêmes avons pu observer que le tellure, quoique filtrant au niveau du glomérule sous une forme diffusible, n'était pas résorbé au même niveau que les carminates et le trypanblau. Nos recherches sur l'athrocytose des colloïdes nous ont montré nettement qu'il existait le long du segment à brosse un gradient de perméabilité apicale; celle-ci est minimale au début et augmente progressivement vers les portions distales. Ainsi, les colloïdes les plus dispersés sont résorbés et fixés dès le début du tube, tandis que le maximum d'athrocytose se décale pour les colloïdes à plus grosse particule (Fig. 3 A, B, C). Ce décalage en sens distal est d'autant plus marqué que le colloïde envisagé est moins dispersé (Gérard et Cordier, 1933 a). Le tube contourné, peut-on dire, constitue par cette propriété curieuse et intéressante, un véritable appareil de mesure de la dispersion.

Nous confirmons donc entièrement l'observation de von Moellendorff et son interprétation, en envisageant toutefois uniquement la perméabilité apicale des segments à brosse tandis que von Moellendorff parle de la perméabilité de la paroi totale du tube; pour cet auteur, en effet, l'athrocytose ne se produit que là où la perméabilité apicale est suffisante pour laisser entrer le colloïde dans les cellules, tandis que la densité cytoplasmique est trop grande pour en permettre la sortie. La propriété athrocytaire du tube contourné est une propriété très primitive. On la

retrouve avec le même caractère dans la néphridie des Oligochètes. L'un de nous (Cordier, 1933) a montré récemment que le segment cilié de l'organe segmentaire du Lombric résorbe et accumule sous forme granulaire tous les colloïdes électro-négatifs injectés dans la cavité cœlomique: colorants colloïdaux acides, bleu de Prusse soluble, solganal, saccharate de fer, oxyde de thorium colloïdal, encre RAL, encre Pelikan 541, chlorophylle, cholestérol. Qui plus est, le gradient de perméabilité mis en évidence dans les néphrons des Vertébrés existe déjà dans la néphridie du Lombric, mais la discrimination que fait le segment cilié entre colloïdes de dispersion diverse est moins perfectionnée. En effet, tous les colloïdes de dispersion haute ou moyenne ont leur maximum d'athrocytose au début du segment cilié

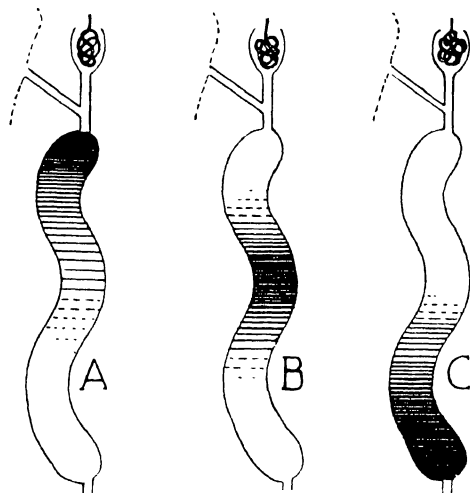


Fig. 3. Athrocytose dans le segment à brosse du néphron ouvert de type II: mésonéphros larvaire de *Discoglossus pictus*. A, athrocytose à maximum initial; colloïdes de haute dispersion. B, athrocytose à maximum moyen; colloïdes de dispersion moyenne. C, athrocytose à maximum terminal; colloïdes de très faible dispersion.

tandis qu'il faut utiliser des colloïdes très peu disperses pour obtenir un décalage du maximum d'athrocytose vers la partie moyenne du segment.

Le pouvoir d'athrocytose du néphron, qui apparaît donc très bas dans la série animale, se retrouve-t-il dans les reins les plus évolués formés de néphrons glomérulaires fermés?

Dans le segment à brosse du néphron fermé des Anoures, on observe régulièrement des inclusions jaunâtres, signalées par de nombreux auteurs et probablement de nature lipopigmentaire. Nous avons observé attentivement la répartition de ces enclaves le long du segment à brosse. Quoique que l'on puisse constater de grandes variations individuelles dans l'abondance et la grosseur de ces inclusions, cette répartition est toujours la même: c'est vers le milieu du segment que l'on rencontre le maximum de grains jaunes (Gérard et Cordier, 1932 a). Ce maximum d'accumulation ne fait-il pas songer étrangement aux images observées après in-

jection de colloïdes de dispersion moyenne dans des néphrons de type I ou II? Et n'est-il pas infiniment vraisemblable que nous sommes en présence de substances colloïdales filtrant par le glomérule et dont le degré de dispersion est légèrement trop faible pour amener une athrocytose à maximum initial?

Même chez les Mammifères, des images d'athrocytose peuvent s'observer. Ainsi, l'on a décrit à diverses reprises chez les Carnivores, des enclaves grasses dans les segments à brosse (Milot, 1927). Nous les avons observées chez de nombreux chats et nous avons pu constater que le maximum de ces enclaves est toujours situé vers la partie terminale de ce segment, contrairement à l'opinion de Peter (1907). Ici encore, nous sommes d'avis que nous nous trouvons en présence d'images normales d'athrocytose.

Si les exemples d'athrocytose normale sont rares chez les Mammifères sains, la pathologie nous en fournit des cas moins sporadiques. Le cas le plus net et le plus intéressant est, à notre avis, celui que l'on observe dans la néphrose lipidique humaine. Cette affection se caractérise, à notre point de vue, par deux aspects particulièrement intéressants : (1) une teneur élevée du sérum sanguin en cholestérol ; (2) une lésion des glomérules se traduisant par une augmentation considérable de leur perméabilité et une albuminurie massive. Les reins de malades morts de néphrose lipidique présentent des tubes contournés chargés d'enclaves cholestériques abondantes. A mesure que les cellules se chargent de cholestérol, on les voit dégénérer, leur noyau se flétrit et enfin elles s'expulsent dans la lumière où elles continueront à se désintégrer. De tels processus s'observent, exactement identiques, lors de l'athrocytose provoquée. Lorsque les cellules du segment à brosse se chargent excessivement de trypanblau par exemple, on les voit, frappées de dégénérescence, tomber dans la cavité tubulaire.

A la lumière des faits observés dans la série des Vertébrés, les images caractéristiques de la néphrose lipidique doivent s'interpréter, non pas comme une dégénérescence lipidique des cellules, mais au contraire comme le résultat d'un phénomène d'athrocytose s'exerçant envers le cholestérol sanguin qui normalement est retenu dans le sang par le filtre glomérulaire, mais qui, à la faveur de la lésion des glomérules, filtre dans les segments à brosse. Cette explication, déjà proposée par Govaerts et Cordier (1928), a trouvé sa confirmation dans une série de recherches que nous avons publiées récemment (Gérard et Cordier, 1933 c). Pour que la nature athrocytaire des images observées fût prouvée, il fallait encore montrer que le cholestérol sanguin se trouve dans des conditions physiques voulues pour que le pouvoir athrocytaire puisse s'exercer envers lui. Pour le démontrer, nous avons injecté le sérum de malades atteints de néphrose lipidique à des animaux, en ayant soin de choisir des espèces à néphrons ouverts ; l'injection à des animaux à néphrons fermés n'aurait pu donner de résultat, puisque l'on sait que le cholestérol sanguin ne traverse pas normalement les endothéliums glomérulaires. Nous avons pu montrer ainsi que l'injection de sérum hypercholestérolique dans la cavité péritonéale de la Salamandre adulte est suivie de l'apparition, au niveau des néphrons ouverts, d'images d'accumulation de cholestérol parfaitement superposables à celles observées dans la néphrose lipidique humaine. On peut même aller plus loin pour

démontrer que ces images sont bien le témoin de l'existence d'un pouvoir athrocytaire. Injectant le sérum de ces malades dans le cœlome du Ver de terre, l'un de nous (Cordier, 1933) obtient, dans le segment cilié des néphridies, des images d'athrocytose de cholestérol identiques à celles observées dans les reins humains. Enfin, ce que la maladie réalise, c'est-à-dire l'augmentation de la perméabilité glomérulaire, nous l'avons pu obtenir par des injections d'ovalbumine. Chez le Crapaud, des injections simultanées d'ovalbumine et de sérum hypercholestérolique sont suivies d'images d'athrocytose de cholestérol, cette fois dans des néphrons fermés (Gérard et Cordier, 1933 c). Les images observées dans la néphrose lipodique ne sont donc pas des lésions spécifiques, mais témoignent de la conservation des potentialités athrocytaires du segment à brosse.

Nous sommes en droit d'affirmer que le néphron des Vertébrés possède dans son segment à brosse des propriétés d'athrocytose très étendues qui se conservent intégralement dans toute la série. Par ce pouvoir le néphrocyte s'identifie à un histiocyte, mais polarisé, car le pouvoir athrocytaire ne s'exerce que par le pôle apical de la cellule. L'apparition du glomérule et la perte de la communication péritonéale limitent singulièrement les possibilités d'expression de cette propriété, mais les expériences réalisées par le chercheur sur l'animal ou par la maladie elle-même chez l'homme, montrent que les cellules rénales ont conservé intactes leurs propriétés d'athrocytose et qu'elles sont prêtes à les exercer, dès que l'occasion leur en est offerte.

VII. LE POUVOIR PHAGOCYTAIRE DU SEGMENT À BROSSÉ.

Au phénomène d'athrocytose se rattache celui de la phagocytose. Ces deux propriétés peuvent être réunies en une seule cellule, l'athrophagocyte de Cuénot. Il importe donc d'envisager si la cellule du segment à brosse est un athrophagocyte.

Le phénomène de la phagocytose implique de la part de la cellule l'incorporation, dans son cytoplasme, de particules "visibles." Ce terme de visible est mal défini, il est passible d'interprétations diverses; pour les uns, ce sera par exemple la plus petite particule visible par le microscope à fond noir; pour d'autres la plus petite particule visible avec le microscope ordinaire (0.1μ). Les tests employés par les auteurs pour mettre en évidence la phagocytose varient aussi de l'un à l'autre; les uns injectent une suspension de carmin; d'autres utilisent de l'encre de Chine; dans l'un comme dans l'autre cas, la grosseur des particules n'aura pas été vérifiée.

C'est pourquoi, tant que l'on aura pas mesuré, par un moyen approprié, la grosseur des particules injectées, il sera impossible de s'entendre sur le terme de phagocytose. Quelle que soit d'ailleurs la limite inférieure que l'on assigne dans une échelle à des particules phagocytées, il est facile d'imaginer une série d'intermédiaires très rapprochés pour arriver à des particules telles qu'on les rencontre dans des solutions colloïdales; d'autre part certains auteurs (Schulemann, de Haan, Höber) pensent qu'un même mécanisme règle athrocytose et phagocytose. Ils désignent parfois l'athrocytose sous le vocable d'ultra-phagocytose. On voit combien sont peu nettes les limites entre ces deux phénomènes. Mais, puisque

nous devons faire un choix dans cette série, nous dirons que la phagocytose commence quand la particule absorbée par la cellule est d'un ordre égal ou supérieur à 0.1μ .

La cellule rénale est-elle capable d'absorber des particules de cette dimension?

Pour le vérifier, nous avons eu recours à l'injection de deux substances à particules facilement visibles et de dimensions nettement supérieures à 0.1μ ; c'est, d'une part, de la mélanine, sous forme d'encre de Seiche, d'autre part, une suspension de cinabre (sulfure rouge de mercure). Chez les têtards de *Discoglossus pictus* (Gérard et Cordier, 1933 d), l'injection intrapéritonéale de l'une ou de l'autre de ces suspensions a été suivie d'absorption de ces substances dans les cellules du segment à brosse des néphrons ouverts, absorption qui témoigne de l'existence d'une véritable phagocytose; ce pouvoir phagocytaire d'ailleurs n'existe pas seulement dans le segment à brosse des Vertébrés, il se retrouve nettement dans le segment cilié de la néphridie du Lombric (Cordier, 1933), qui se montre capable d'absorber et de fixer l'encre de Seiche introduite dans la cavité coelomique.

La cellule rénale du segment à brosse n'est donc pas seulement un athrocyte, elle peut fonctionner aussi comme athrophagocyte.

VIII. ATHROCYTOSE ET RÉSORPTION DE SUBSTANCES DIFFUSIBLES.

Il nous reste à examiner si le phénomène d'athrocytose qui, répétons-le, implique la résorption de substances colloïdes, leur concentration et leur accumulation granulaire, et leur rétention énergétique, est identifiable ou superposable aux processus de résorption de substances dissoutes postulés par la théorie de Ludwig-Cushny. Ce qui différencie essentiellement les deux processus, c'est que l'athrocytose est liée à la nature colloïdale des substances absorbées et s'accompagne d'une rétention de celles-ci. Les colloïdes que l'on rencontre dans les athrocytes, tant rénaux que les autres (tissu réticulo-endothélial), ne sont pas en voie d'élimination. C'est à tort que de très nombreux auteurs observant, après injection de carminate d'ammoniaque par exemple, des inclusions carminiques dans les cellules rénales, parlent d'images d'"élimination." Bien au contraire ces images signifient que l'organe rénal agit dans ce cas comme un rein d'accumulation et nullement un émonctoire vrai.

La résorption physiologique des substances diffusibles ne s'accompagnerait pas d'une telle rétention. Les substances résorbées, chlorures, glucose, etc., seraient restituées au milieu intérieur.

Il n'est cependant pas exclu qu'elles ne puissent se concentrer dans le cytoplasme des segments à brosse après leur résorption. Les études de physiologie générale et spécialement de physiologie végétale nous ont appris en effet que la cellule a le pouvoir d'absorber des ions du milieu environnant et de les concentrer au sein de son cytoplasme à des taux souvent impressionnants. Le suc de *Valonia* ne renferme-t-il pas 40 fois plus de K que l'eau de mer dans laquelle vit cette algue (Osterhout, 1927)? Les hématies ne contiennent-elles pas 13 fois plus de K que le plasma sanguin?

Il est fort possible que la résorption physiologique n'utilise qu'une partie des potentialités de la cellule rénale. La résorption de substances dissoutes s'accompagne peut-être de concentration dans les cellules mais non d'une floculation et d'une rétention plus ou moins durable. Quoi qu'il en soit, au point de vue histophysiologique, la question de la résorption des substances normalement présentes dans le filtrat glomérulaire doit rester sans réponse tant que nous ne disposerons pas de moyens techniques suffisamment dignes de confiance pour déceler ces substances dans les tissus et cellules. Dans l'état actuel de nos connaissances chimiques, on ne peut guère nourrir l'espoir d'arriver bientôt à des méthodes histologiques capables de satisfaire à ce desideratum. Cependant les méthodes physiologiques de micro-pipetage (Richards et collaborateurs) nous apportent la preuve de la résorption de substances diffusibles.

IX. CONSIDÉRATIONS PHYLOGÉNÉTIQUES.

De l'ensemble des considérations précédentes, nous concluons que le néphron des Vertébrés, si remarquablement uniforme dans sa structure, présente également dans sa physiologie une unité frappante. Phylogénétiquement, le néphron primitif théorique serait évidemment un néphron ouvert aglomérulaire dont on ne rencontre plus d'exemples chez les Vertébrés, mais qui est encore abondamment représenté chez les Invertébrés (nombreux Annélides).

Son évolution chez les Vertébrés se complique par l'apparition d'un peloton capillaire artériel, qui, dans les types les plus primitifs (type I), est simplement suspendu en regard des néphrostomes et qui plus tard s'incorpore au néphron lui-même sous forme de glomérule de Malpighi. Celui-ci réalise un rapport intime entre le néphron et le système clos constitué par l'appareil vasculaire, la communication cœlomique pouvant d'ailleurs persister (type II). Enfin, dans les types les plus évolués, cette communication se perd et le néphron débute par le glomérule de Malpighi: à ce niveau, sa cavité (cavité capsulaire) n'est séparée du système vasculaire sanguin que par une barrière réduite à l'endothélium vasculaire, doublé d'un réseau péricytaire.

Quelle est la position qu'il convient d'attribuer, dans ce schéma évolutif, au néphron fermé aglomérulaire (type IV)?

Nous ne pouvons le considérer comme primitif; nous serions plutôt enclins à croire qu'il est le résultat d'une mutation caractérisée par une absence de développement des artères rénales; les glomérules ne se formant pas, le néphron ne s'est différencié qu'en une seule direction: la sécrétion, d'où absence du segment à bâtonnets. Quant à la communication cœlomique, elle ne se développe pas, et à ce point de vue, le rein aglomérulaire des Téléostéens ne se distingue pas du rein glomérulaire, dans la même classe de Vertébrés.

Hâtons-nous d'ajouter que, dans ce schéma, nous n'envisageons que le néphron des Vertébrés et non celui des Chordés en général. Ainsi, dans le phylum des Chordés, le rein de l'*Amphioxus* occupe une place tout-à-fait à part, étant formé de néphrons à solénocytes, tels que l'on en rencontre chez les Invertébrés inférieurs.

X. RÉSUMÉ.

1. Les néphrons des Vertébrés peuvent se classer en néphrons ouverts et néphrons fermés, selon qu'ils présentent ou non une communication directe avec la cavité péritonéale, par l'intermédiaire d'un néphrostome.

2. Le glomus et le glomérule sont des appareils filtrants laissant passer, non seulement les substances diffusibles, mais encore les colloïdes de haute dispersion.

3. Le segment à brosse jouit de propriétés résorbantes, s'exerçant aux dépens de substances filtrant par le glomérule. Cette propriété est nettement mise en évidence par l'injection de substances colloïdales très disperses qui apparaissent dans les cellules de ce segment lorsque le glomérule fonctionne normalement et qui n'y pénètrent plus lorsque la fonction glomérulaire est expérimentalement supprimée.

4. Le segment à brosse jouit d'un pouvoir athrocytaire, caractérisé par l'absorption, la concentration et la rétention sous forme granulaire de *tous* les colloïdes portés au contact du pôle apical de ses cellules. L'athrocytose de substances colloïdales de dispersion moyenne ou faible ne peut évidemment s'observer que dans les néphrons ouverts, ou encore dans les néphrons fermés dont la perméabilité glomérulaire a été pathologiquement augmentée (néphrose lipoidique).

5. Le segment à brosse est phagocytaire, par son pôle apical (absorption de mélanine et de cinabre).

6. Il existe, dans le segment à brosse, un gradient de perméabilité apicale des cellules. Cette perméabilité est minimale au début du segment et augmente progressivement en sens distal. L'existence de ce gradient est démontrée par le décalage en sens distal, du point d'athrocytose maximale pour des colloïdes de dispersion diverse; ce décalage est d'autant plus marqué que le degré de dispersion est plus faible.

7. L'athrocytose de substances colloïdales diffère de la résorption des substances diffusibles en ce qu'elle s'accompagne d'une rétention prolongée des substances résorbées.

8. Le segment à brosse jouit en outre d'un pouvoir émonctoire, grâce auquel des substances de déchet peuvent être directement éliminées au dehors, sans filtration préalable par le glomérule. Cette fonction émonctoire, évidente dans les néphrons aglomérulaires, peut être démontrée expérimentalement par suppression de la fonction glomérulaire chez l'Anoure: l'élimination de l'acide urique continue à se faire dans ces conditions.

9. Le segment à bâtonnets possède un pouvoir de résorption d'eau, démontré par les images obtenues après injection d'acide urique ou de nitrate d'urane.

10. Le néphron primitif du Vertébré est un néphron ouvert. L'apparition du glomérule s'accompagne de la perte de la communication péritonéale.

XI. SUMMARY.

1. Vertebrate nephrons can be classified into open and closed according as they have or have not direct communication with the peritoneal cavity through the intermediary of a nephrostome.
2. The glomus and the glomerulus are filters which allow not only diffusible substances to pass but also highly dispersed colloids.
3. The "segment à brosse" [proximal convoluted tubule] possesses a resorbant function with respect to substances filtering through the glomerulus. The property in question can be clearly demonstrated by the injection of highly dispersed colloids, which reappear in the cells of this segment when the glomerulus functions normally, but no longer penetrate into them when the glomerular function is experimentally inhibited.
4. The "segment à brosse" has an athrocytary function, characterised by the absorption, concentration, and retention in granular form, of all colloids brought into contact with the apical pole of these cells. Athrocytosis of colloids of medium or feeble dispersion can, of course, only be seen in the case of open nephrons, or of closed nephrons the glomerular permeability of which has been pathologically augmented.
5. The "segment à brosse" acts phagocytically through its apical pole, absorbing melanin and cinnabar.
6. There exists a gradient in the apical permeability of the cells in the "segment à brosse." This permeability is at a minimum at the beginning of the segment and increases progressively in the distal direction. The existence of this gradient is shown by a shift in the distal direction of the point of maximal athrocytosis for colloids of varying dispersions: the shift is the greater the lower the degree of dispersion of the colloid.
7. Athrocytosis of colloids differs from the resorption of diffusible substances in that it is accompanied by a prolonged retention of the substances resorbed.
8. The "segment à brosse" also possesses an excretory function by which waste matters can be directly excreted without previous filtration through the glomerulus. This excretory function, clearly evident in nephrons lacking glomeruli, can be demonstrated experimentally by the suppression of the glomerular function in *Anura*. Under these circumstances uric acid continues to be excreted.
9. The "segment à bâtonnets" [distal convoluted tubule] has the capacity of resorbing water. This can be demonstrated by the injection of uric acid or of uranium nitrate.
10. The primitive vertebrate nephron is open. The appearance of the glomerulus is accompanied by a loss of the communication with the body cavity.

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THE INTERPRETATION OF PHYLLOTAXIS

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WE are glad that Priestley and Scott (1933), in a recent article in this periodical, have ranged themselves with those who consider that the positions in which new leaves arise are determined by the positions of those already present; for we claim to have produced direct evidence (1931, 1933) showing that the truth lies with the theories of this kind. But as to the exact manner in which the positions of the new leaves are determined by those of the older ones, several points in the article by Priestley and Scott seem to us to need comment.

Some of these points concern Hofmeister's rule. The authors write as if Hofmeister's rule were that the next centre of vigorous growth must be established as far as possible from the previous one (p. 243). Again they write (p. 251) that, for Fibonacci phyllotaxis, a simple extension of Hofmeister's rule would be that "each successive primordium tends to be as nearly opposite its predecessor as possible, allowing for the fact that the primordium two before it in origin... is also as nearly as possible opposite to this same predecessor and is still growing." If we rightly understand this statement, it means that (in Fibonacci phyllotaxis) each new leaf tends to arise as far as possible from both the two previous leaves.

But there is no need to "extend" Hofmeister's rule in order to make it apply to all the systems of Fibonacci phyllotaxis: for Hofmeister himself considered the higher systems as well as the lowest system, and formulated his rule so as to make it apply to them all (1868, pp. 488 *seq.*). Moreover the "extension" offered by Priestley and Scott is untenable, for reasons given below.

Hofmeister began his famous chapter on phyllotaxis with the statement, "It is a fundamental experience that new leaves (or side-shoots) arise in those positions on the stem apex (or axis) which are furthest from the side edges of the bases of the most closely neighbouring leaves that are already present" (1868, p. 482). By the expression "the most closely neighbouring leaves" he meant the leaves that form the uppermost cycle or ring round the apex, as is clear from his subsequent discussion. He went on to illustrate this rule with examples of various phyllotaxis systems; and he made it clear that it is only in "distichous" or two-rowed phyllotaxis that the position of the new leaf depends on that of the immediately previous one alone (p. 485). For, as he pointed out, in this system each leaf covers an arc of more than 180° , so that the new leaf arises above the gap between the two edges of the previous one, on the opposite side of the apex (p. 485). He further pointed out that in higher systems of phyllotaxis, in which each young leaf covers an arc

of less than 180° , there are at any time two or more gaps between the leaves of the uppermost cycle round the apex. In order, therefore, to include these systems also under his rule, he formulated it as follows: "The new leaf arises above the one gap, or, if several gaps are present, above the widest gap¹" (p. 488).

Now when there are several gaps between the leaves of the uppermost cycle, or (as comes to the same thing) when there are several leaves in that cycle, it may be expected that the position of the next leaf will depend most directly on the positions of those two older leaves which lie on each side of the gap above which it will arise; and evidence will be given below to show that this expectation is correct. But these two older leaves are by no means always the two immediately previous leaves, upon which Priestley and Scott (pp. 251, 256-7) state that the position of the next leaf mainly depends. It is true that in the system with contact parastichies $1+2^2$ the next leaf will arise above the larger of the two gaps between the two immediately previous leaves: for in this system there are only two leaves in the uppermost cycle. But in the system with parastichies $2+3$, which is very common, there are at any time three leaves in the uppermost cycle, and the next leaf, which may be called x , will arise *not* above the gap between the two previous leaves $x-1$ and $x-2$, but above the gap between the leaves $x-2$ and $x-3$, with which it will make contact. Similarly in the system $3+5$, there are five leaves in the uppermost cycle, and the next leaf " x " will arise above the gap between the leaves $x-3$ and $x-5$. In order to see that this is so, one need only look at bud sections of plants with these systems (e.g. van Iterson, 1907, Figs. 44, 47) or at theoretical diagrams (*ibid.*, Plate 10).

There is therefore no reason to expect that the two immediately previous leaves will be of special importance in determining the position of the next leaf except in the system $1+2$ (and also the "decussate" system $2+2$), in which there are only two leaves in the uppermost cycle. Indeed in *Lupinus albus*, which has a $2+3$ system³, our experiments (1931, pp. 17, 21; 1933) show that the leaf $x-1$ is of much less importance (if any) in determining the position of leaf x than are the leaves $x-2$, $x-3$, between which the leaf x will arise, and with which it will make contact. For by operating in certain ways on the part of the apex from which the next leaf (which we called I_1) was due to arise, we caused very little change, if any, in the position

¹ "Oberhalb der einzigen, oder wenn mehrere vorhanden der breitesten Lucke tritt das neu entstehende Blatt...hervor." See also p. 508.

² By the statement that a phyllotaxis system possesses $m+n$ contact parastichies, it is meant simply that near the apex one can see m curved paths, along which the leaves are in contact, winding obliquely in one direction, and n curved paths crossing these and winding in the other direction. These two sets of contact parastichies are the only ones, if each leaf touches only two older leaves below it. But in some plants each leaf touches three older leaves below, and there are then three sets of contact parastichies, two sets winding one way (with different steepness) and one set the other way. The system then possesses $l+m+n$ contact parastichies. We give reasons later for considering that the Schimper-Braun "fractional" classification cannot be applied to phyllotaxis systems near the apex.

³ To speak strictly, the system is $1+2+3$, since the edges of the stipules of successive leaves just touch along the genetic spiral. But the *central* part of each leaf x arises in the gap between the leaves $x-2$ and $x-3$, and makes contact with these leaves only. Consequently it can be understood how it was that in the experiments the stipular contact with leaf $x-1$ was of much less importance in determining the position of the central part of leaf x .

of I_2 (the next leaf after I_1), but big changes in the positions of I_3 and subsequent leaves. Also similar operations on P_1 (the youngest of the leaves already visible) caused little change, if any, in the position of I_1 , but considerable change in the positions of I_2 and I_3 .

Here we may remark incidentally that at one point Priestley and Scott (p. 244) advance the theory that the position of a new leaf depends not only on the positions of the older leaves, but also on those of the leaves *younger* than it (which do not yet exist when its own position is determined). This theory would necessitate the very difficult assumption that a leaf already determined can be somehow displaced by those that are determined after it. The theory is inconsistent with the authors' own remarks on pp. 256-7, where they state that the position of each new leaf is determined mainly by the two most recent of the previous leaves, and there is absolutely no evidence that supports it.

Since the work of Hofmeister (1868) it has been generally admitted, as a fact of observation, that each new leaf arises in the largest gap between the previous ones, in most species at least. But the question remains, how is this fact to be explained? Priestley and Scott consider that each new leaf tends to arise as far as possible from the previous leaf (p. 243), or (in Fibonacci phyllotaxis) from the two previous leaves (p. 251), with which it is in some sense "competing." It is indeed very natural to assume some such tendency (except that in $2+3$ and higher systems, as already pointed out, it is not only the two previous leaves which must be considered), and a similar assumption has been made by Schmucker (1933). But is such an assumption really necessary?

With regard to this question, Priestley and Scott give no account of the very valuable and important work of van Iterson (1907), who succeeded in explaining most of the main facts of phyllotaxis without postulating any tendency for the next leaf to arise as far as possible from previous leaves. One of the simple facts of observation on which his theory is based, is that the new leaves arise in the largest (or as he prefers to say "larger") gaps between the previous ones; but, strictly speaking, he is bound to assume further that the positions of the new leaves are actually determined by the positions of these gaps. We claim now to have produced direct experimental evidence showing that they are indeed so determined (1931, 1933). Now in order to explain how it is that each successive new leaf is determined in the largest gap between the previous ones, one need only suppose that, before a leaf can be determined, there must be available on the surface of the apex a space of some minimum size at some minimum distance below the extreme summit or "growing-point." For the largest gap between the previous leaves will, as a general rule, be the one in which, through the growth of the apex, the minimum space necessary for leaf-formation will first become available. A conception of this kind seems to us necessary for the further working out of van Iterson's theory, and we have tried elsewhere to develop it more fully (1931, p. 16; 1933, pp. 360, 396 *seq.*).

There is therefore no need to assume that the leaf that is arising tends in any way to be repelled away from the older leaves, and, further, the available evidence is against this assumption. For, on this assumption, it could not be understood how

it is that in *Lupinus albus*, as already pointed out, the position in which leaf x will arise depends much more on the positions of leaves $x-2$ and $x-3$ than on that of leaf $x-1$. We have also found that in our experiments the outlines of the wounds which we made on the apex acted in practically the same way, in determining the positions of subsequent leaves, as did the young leaves round the apex. Now if the young leaves acted by means of any tendency to repel the subsequent leaves, this would be difficult to understand.

At various points in their article (e.g. pp. 256, 262), Priestley and Scott briefly discuss questions which van Iterson discussed very thoroughly. These are concerned with the conditions on which depend the different systems of phyllotaxis found in different plants. It is indeed one of the main conclusions of van Iterson's book (1907) that the phyllotaxis of any plant depends in the main on two things, firstly the relative sizes of the young leaves and the apex, and secondly the way in which the system started. In the mathematical first part of his book he investigated the geometry of the various possible "similar systems of touching circles" on the surfaces of cylinder, cone and plane, and showed that when these circles cover certain fractions of the circumference, only certain contact systems are possible. These results are summarised graphically in Pl. 2, fig. 2. In the botanical second part he made the hypothesis that the insertions of the young leaves form a "similar system of touching circles" on a cone surface in the sense previously defined. He tested this hypothesis by examining bud sections of many species, and observing whether the appearances of the sections and the relations between the contact systems and the ratios of size of leaf to size of apex are those which, if the hypothesis is correct, are to be expected on the basis of the geometrical investigation. He concluded that, for the species investigated, the hypothesis is correct, but pointed out that, in various other species, the young leaves are probably not circular in outline (p. 295).

In order to explain the causes through which a phyllotaxis system, once established, is continued, he postulated, as already stated, that the new leaves arise in the larger gaps between the previous ones. But in order to understand how the various phyllotaxis systems are first established, and how it comes about that the Fibonacci systems are so common, it is necessary to consider how these systems originate in seedlings and axillary shoots. Concerning these questions, reference may be made to van Iterson's observations and theoretical considerations (1907, pp. 273-89, 253-73). His treatment of all these questions is to some extent based on that of Schwendener (1878) and other earlier workers.

Priestley and Scott make some remarks which imply that the ratio between size of young leaf and size of apex is important as a factor on which the phyllotaxis depends (p. 256), but in this connection they do not refer to van Iterson (1907), nor to Hofmeister (1868) or various subsequent workers, who all recognised the importance of this ratio.

Here we may remark also that the conclusion of Priestley and Scott (pp. 256-7), that the genetic spiral is only a secondary phenomenon, is implicit in the work of all those who have followed Hofmeister's lead, for instance Schwendener (1878),

van Iterson (1907), Schoute (1913). It is also pointed out by Church (1902, pp. 106-7).

Some comment seems to be needed also on the Schimper-Braun "fractional" classification of phyllotaxis systems which Priestley and Scott employ. For it is disappointing to find that an attempt is still made to use this classification, even after the very thorough manner in which Church (1901) criticised it and showed the difficulty of applying it. Priestley and Scott write (p. 243): "The usual method for describing the leaf arrangement... is to express it in terms of the fraction of the circumference of the axis between one leaf and the one immediately succeeding. Thus the simplest types of phyllotaxis are $1/2$ and $1/3$, and other more complex types are $2/5$, $3/8$, $5/13$, etc." They then proceed to discuss phyllotaxis systems at or near the apex in terms of this classification. But clearly this classification could not usefully be applied to the phyllotaxis systems that are actually found near the apex, unless it were found on investigation that near the apex the divergence angles between successive leaves in various species did really fall into separate groups, having values of approximately $1/2$ of 360° , $1/3$ of 360° , $1/5$ of 360° , etc.

Now concerning the first of these groups, of angle 180° , there is no difficulty: for this angle is found in the familiar "distichous" phyllotaxis. Also some plants show divergence angles of 120° , or only a little more, e.g. various Cyperaceae and mosses. But as to the other groups, $2/5$ or 144° , $3/8$ or 135° , etc., there is no evidence that the divergence angles found near the apex in various species tend to fall into separate groups with approximately these values, and it is unlikely that they do so. Such measurements as are available do not indicate that in the neighbourhood of the Fibonacci angle (137.5° approx.) there is anything but a continuous distribution of the mean divergence angles of different species, with a maximum frequency not far from the Fibonacci angle itself¹. Near the apex, therefore, so far as is known, phyllotaxis systems of $2/5$, $3/8$, $5/13$, etc., as separate classes, do not exist: and for this reason discussions of such supposed phyllotaxis systems and constructions to illustrate a transition from $2/5$ to $3/8$ phyllotaxis, such as Priestley and Scott give (p. 255), have no application to anything that has been found in shoot apices.

In order to classify phyllotaxis systems, one must use classes into which these systems are actually found by observation to fall. The classification by the numbers of the parastichy curves seen near the apex, which was used by Church (1901) and was explained above, is useful and easy to apply. Van Iterson classifies by means of the numbers of steps or "plastochrons" intervening between the times of origin of the leaves that make contact. This classification gives numerically the same result as that of Church, except when m and n have a common factor greater than 1. It has the advantages that it is based on the contacts of the young leaves close to the apex only, and that one can often see with which leaves the youngest visible leaf makes contact, even when the parastichies cannot easily be counted.

It should also be noted that though the "fractional" classification cannot be

¹ There are, however, some indications that angles a few degrees above the Fibonacci angle are probably more common than angles a few degrees below it (see Church 1904, p. 430, van Iterson 1907, p. 208 and p. 212).

applied to phyllotaxis systems near the apex, yet it often *can* be applied to the systems lower down the stem. For lower down the stem, the divergence angles, as is commonly agreed, tend to approximate to the angles corresponding to the Schimper-Braun fractions, as a result of slight secondary torsions: and it is probably for this reason that the fractional classification has remained in use for so long. It is indeed clear that Priestley and Scott (p. 253) contemplate applying the fractional classification to phyllotaxis systems as seen well below the apex, though they are discussing the origin of these systems at the apex.

An explanation of these secondary torsions was advanced by Schwendener (1883) and confirmed by Teitz (1888) with various observations. It is accepted by van Iterson (1907, pp. 227 *seq.*) and Schoute (1913, p. 287). It is briefly that the leaf traces usually connect up so as to form strands which, near the apex, wind obliquely round the stem. These strands sometimes form along the so-called "orthostichies," which near the apex are slightly curved. But lower down, the elongation of the stem sets up tensions in these strands, which tend to pull them straight. As the strands straighten, they force the whole stem to twist slightly, until the leaves which are situated along the strands come to be situated in vertical rows. And when this happens, the divergence angles between these old leaves reach values corresponding to some one of the Schimper-Braun fractions.

It seems to us that discussions of the factors determining the positions of leaves are not likely to help matters much except in so far as they are based on observations or experiments on shoot apices. But there is a great deal of relevant observational work that needs to be done, even apart from the method of direct experiment which is now open.

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CYCLIC REPRODUCTION, SEX DETERMINATION AND DEPRESSION IN THE CLADOCERA

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CONTENTS.

	PAGE
I. Introduction	139
II. Cyclic reproduction and sex determination in the Cladocera; their explanation by a hypothesis of depression	140
III. Other experimental investigations into the biology of the Cladocera, the results of which support the depression hypothesis	144
IV. Investigations in nature which support the depression hypothesis	155
V. Cyclic reproduction, sex determination and depression in the Rotifera: a comparison with similar phenomena in the Cladocera	159
VI. Remarks on peculiarities connected with the state of depression	167
VII. Summary	172
References	173

I. INTRODUCTION.

OF late years not a few works have been written on the sex determination and the conditions underlying the transition from parthenogenesis to gamogenesis in the Cladocera. Among the most important of these works are a series of papers by Banta and Brown (1929-30) and a paper by A. Tauson (1930), all of which have considerably increased our knowledge in regard to the cyclic reproduction of these animals, and more fully elucidated several biological conditions which were formerly imperfectly known or were a matter of controversy. Thanks to this newly acquired knowledge, we are now also in a much better position to form an opinion of the earlier literature, in which highly contradictory views are put forward concerning the sex determination and the cyclic reproduction in the Cladocera.

Almost contemporaneously with the appearance of the above-mentioned works, the present writer also published a paper on the sexuality of some Cladocera, viz. the genus *Daphnia* (1931). In order to explain the transition from the exclusive production of females to the production of males as well, and also the transition from parthenogenesis to gamogenesis, I therein set forth a so-called depression hypothesis to be mentioned in detail below. The purpose of the present article is in the first place to draw certain comparisons between my results—especially the depression hypothesis—and those arrived at in the above-mentioned recent works on the Cladocera, in order, if possible, to extract a general doctrine, a common

denominator. At the same time it will, of course, be only just to examine the results set forth in earlier works on the same problem with a view to ascertaining whether they may be said to support or to contradict the recent results. There can be no doubt that such an investigation is much needed. Both in the earlier and in the recent literature mention is made of a great many experiments and observations in nature, showing that certain external factors—temperature, quantity of nourishment, chemical factors, etc.—have a sex-determining influence on the Cladocera. But can these factors be regarded from the same point of view? Why is it, for instance, that a certain small quantity of nourishment and a certain degree of acidity have similar effects on the reproduction in the Cladocera? It is not easy to see how such dissimilar factors can produce such uniform sex-determining effects. Why is this so? What is it that these sex-determining factors have in common?

Biological investigations seeking to answer such questions have a value of their own and are as important as cytological studies on sex determination; for the knowledge of a possible cytological mechanism which regulates the determination of sex in cyclic reproduction is not sufficient for the full comprehension of reproduction and sex determination. At the same time it is necessary to find out why sometimes parthenogenetic and sometimes gamogenetic eggs mature in the ovaries, and why the parthenogenetic eggs sometimes prove to be male eggs and sometimes female eggs. Perhaps it may even be permissible to maintain that these questions represent the most important aspect of the problem of sex determination; Goldschmidt (1920, p. 201) expresses this in the following words: "... das Hauptproblem kommt doch schliesslich auf die Aufdeckung der Faktoren hinaus, die Reifeteilung und Parthenogenese beeinflussen können."

II. CYCLIC REPRODUCTION AND SEX DETERMINATION IN THE CLADOCERA; THEIR EXPLANATION BY A HYPOTHESIS OF DEPRESSION.

Between the cycle of reproduction of the daphnids on the one hand and their life conditions on the other there is a perfect harmony, an agreement. Under favourable conditions the animals propagate their species parthenogenetically and quickly, and in that case they only produce females; when unfavourable conditions intervene (cold, drought, etc.), their reproduction is by male-female (gamogenetic) generations, they form resting eggs and die off.

Now, how is this to be explained? What is cause and what is effect? Is it the existing conditions or external circumstances that directly determine whether the mother animals are to give birth parthenogenetically to male or female offspring? Are external conditions also responsible for the production of parthenogenetic eggs or resting eggs requiring fertilisation?

Or were the earlier investigators—for instance Weismann (1876-9) and several others—right in supposing that the life cycle of the daphnids is subject to quite definite rules, so that they first pass through a certain number of parthenogenetic generations, which are then automatically followed by gamogenetic generations producing fertilised resting eggs? May the whole process thus be compared to a

clockwork which, once wound up, goes on according to established laws? I have undertaken a series of investigations in order to throw light on these problems, and in the following pages some of my laboratory experiments and investigations in nature are summed up.

1. It has proved possible in various ways to cause *Daphnia* females that reproduce parthenogenetically in nature and only produce females, to produce males and resting eggs when kept in cultures. One of the methods is the so-called crowding method, that is to say, a crowding together of parthenogenetic females in a rather small space of water (Grosvenor and Smith, 1913; Banta and Brown, 1923). A proportion of the females then change over and become gamogenetic. This method very closely imitates conditions in nature, where gamogenesis replaces parthenogenesis at times when the number of individuals per litre is large.

Among the crowding experiments, the following may be mentioned as an example: In the middle of the summer a number of females of *Daphnia cucullata* were caught in a lake. They were all in parthenogenesis, and—as might be expected—all the young animals produced in the lake were females. The captured animals were taken into the laboratory and placed in very small aquaria under crowding conditions, which produced a certain state of depression in them. It then turned out that under these conditions their young consisted not only of females, but also of a large number of males. Thus the life cycle was altered: the males had been brought into the world long before it would have happened in nature. At the same time some of the females started producing ephippia with resting eggs, which must be fertilised before they can develop (Fig. 1).

Another experiment showed a similar result, but was, if possible, still more convincing. It was a case of females—so-called ex-ephippio females—which had themselves been hatched from ephippia in the spring. They should, therefore, normally have been the originators of a long series of parthenogenetic generations of females; but in the crowding cultures they were compelled to produce both males and ephippia with resting eggs (Fig. 2).

In a similar way and with the same results experiments have been carried out with other generations of *Daphnia* whose reproduction in nature would have been parthenogenetic. Females which have wintered, the offspring of wintering females, the first generation after the ephippia, summer and winter generations, have all been compelled to gamogenesis. Consequently, the doctrine advanced by Weismann (1876-9, p. 428), that the gamogenetic individuals in the cycle of the Cladocera are limited to definite generations and broods, cannot be accepted.

2. Through the preceding experiments, carried out in the laboratory, and through similar experiments, it has been proved that if certain daphnids are made liable to depression by being exposed to unfavourable conditions, gamogenetic reproduction *may* be induced in them. But this does not necessarily mean that because a depression may give rise to a sexual period, such a period may not be due to internal causes, without the animals having been exposed to unfavourable conditions; this might possibly be the case in nature.

If in nature the sexual periods are due to internal causes, one would not be

justified in looking for symptoms of depression in the animals during these periods; if, however, unfavourable conditions are also instrumental in producing gamogenesis in nature, it is possible that signs of depression may occur in the animals at the same time. The question therefore arises: In the *Daphnia* population in nature, is the transition from parthenogenesis to gamogenesis accompanied by a state of depression in the population in question, or is this not the case?

In order to answer this question it will be necessary to point out one or more indications which can show the existence of a possible depression.

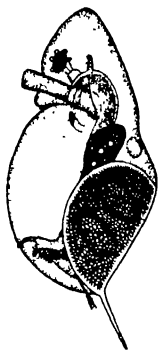


Fig. 1.

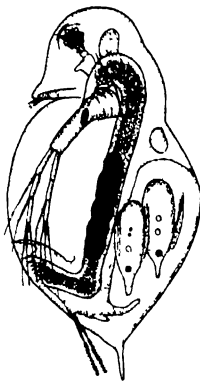


Fig. 2.

Fig. 1. *Daphnia cucullata* female with an ephippium and two resting eggs. Animal drawn July 12th, 1929; it is a summer form which would have had parthenogenetic propagation in nature. In a crowding experiment it has been forced to form an ephippium containing a resting egg which must be fertilised. $\times 32$.

Fig. 2. Ex-ephippium female of *D. cucullata*. The female has lived in a crowding culture. She has thrown off an ephippium, as is plainly evidenced by the outline of the back, the latter showing a rather distinct break instead of forming a smooth curve. After discarding the ephippium, the female has produced a parthenogenetic brood of two young ones, which were almost full-grown when the drawing was made, and had high helmets. They subsequently proved to be males. That the ex-ephippium females will again pass over to gamogenetic reproduction after this brood is evident from the appearance of the ovary with its compact dark mass representing a resting egg in process of formation. $\times 32$.

It is a fact well known to everybody who has kept daphnids in cultures that these animals react to favourable conditions by producing large parthenogenetic broods, and to injurious influences (insufficiency of food, low temperatures, impurities in the water, and so on) by having small broods. Kuttner (1909), for instance, has found twenty to thirty eggs in well-nourished individuals of *Daphnia pulex*, and only two to four to the brood in animals suffering from hunger. Studies in nature furnish similar examples, proving that the size of the broods must be considered dependent on the conditions of life. The size of the parthenogenetic broods may, therefore, serve as a visible measure of the influence of life conditions.

Accordingly the average number of parthenogenetic eggs was determined in a series of *Daphnia* populations at different seasons, both at times when the species in question was in pure parthenogenetic reproduction and also at times when it was

in gamogenesis. The results were given in curves, which on the whole showed a good agreement: two of them are reproduced in Figs. 3 and 4. The curves showed that, immediately before the beginning of the sexual periods, a considerable diminution in the average number of eggs in the parthenogenetic females manifests itself.

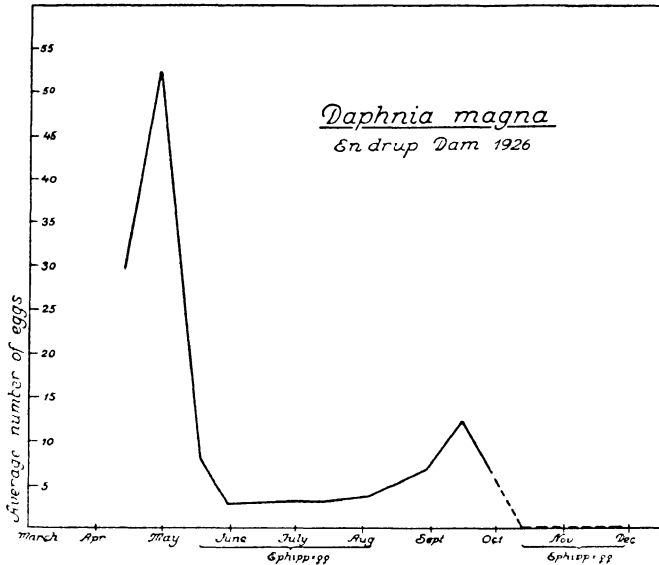


Fig. 3. The average number of eggs carried by the egg-bearing parthenogenetic females of *D. magna* is given as ordinates and the months as abscissae; the periods during which ephippial females occur (Ephipp. ♀♀) are indicated. As will be seen, the curve plainly shows that before each period of ephippia production the average number of eggs produced by the parthenogenetic females falls considerably.

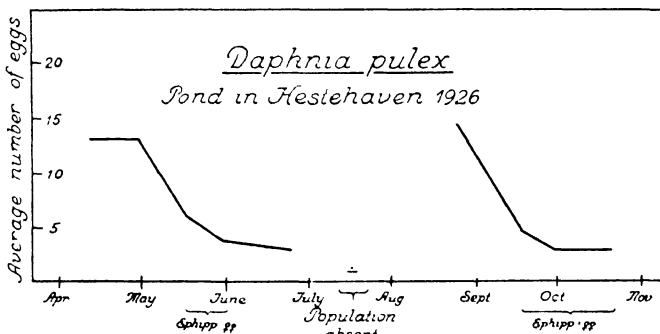


Fig. 4. The curve shows that before the spring and autumnal period of ephippial formation there is a marked fall in the average number of parthenogenetic eggs of *D. pulex*.

This diminution cannot be accounted for by a corresponding diminution in the average size of the egg-bearing females themselves. It can, therefore, only be interpreted as a manifestation of a state of depression in the *Daphnia* population in question during the transition from parthenogenesis to gamogenesis.

Conditions among the daphnids were also examined in another way. The number of fat globules to be found in the ovary and surrounding tissue of *D. cucullata* and *D. longispina* shows seasonal variations. As it might be supposed that these fat globules would furnish some information in regard to the state of nourishment of a population, if a sufficiently large number of individuals were examined, such an examination was carried out at different stages of the cycle of *D. cucullata* and *D. longispina*. By means of a camera lucida a large number of figures were drawn showing the fat globules in living, recently captured animals, the result being a clear picture of the state of the population in this respect at the given moment. The same was done at other seasons, and a comparison thus rendered possible.

As stated, the examination was carried out on *D. cucullata* and *D. longispina*. The former was monocyclic with a sexual period in the autumn. At the incidence of the sexual period and while it lasted, the production of fat globules in the parthenogenetic females was less (Fig. 5 A, B) than in periods of vigorous parthenogenesis (Fig. 5 C, D). *D. longispina* had a sexual period in the early summer. Parthenogenetic females living during the sexual period show a smaller production of fat globules (Fig. 5 E, F) than those living previously during the period of pure parthenogenesis (Fig. 5 G, H). Hence, in both species the populations are probably subject to a state of depression during the sexual period.

Conclusion. Taken in conjunction, the two observations, (1) that a state of depression produced by unfavourable external conditions in *Daphnia* females kept in cultures, causes gamogenetic propagation (point 1 above), and (2) that gamogenesis in nature is accompanied by a state of depression in the populations in question (point 2 above), go to prove the correctness of the hypothesis that in nature the transition from parthenogenesis to gamogenesis is caused by the influence of unfavourable external conditions. The unfavourable external conditions cause states of depression in the females and thereby the change in the mode of reproduction (Berg, 1931).

III. OTHER EXPERIMENTAL INVESTIGATIONS INTO THE BIOLOGY OF THE CLADOCERA, THE RESULTS OF WHICH SUPPORT THE DEPRESSION HYPOTHESIS.

In the abundant literature dealing with the reproduction in the Cladocera, mention is made of a considerable number of experiments undertaken for the purpose of throwing light on the influence of external life conditions upon the mode of reproduction and the sex determination of these animals. The results arrived at have sometimes been thought to favour the view that external conditions have no influence on the reproduction, while at other times they have been interpreted as evidence of the opposite—and finally there are a number of investigators who occupy an intermediate position between the two points of view. These latter are of opinion that external conditions may exercise some influence, but that internal, hereditarily fixed conditions also have a determining influence on the cycle of reproduction.

Whichever of these conclusions the authors may have drawn from their experiments, we shall now see that a number of the experiments in themselves clearly

support the above established hypothesis to the effect that the change from parthenogenesis to gamogenesis is caused by a state of depression in the animals, and that this depression is due to external conditions. For at the same time as parthenogenetic or gamogenetic reproduction was observed in the course of the experiments, other

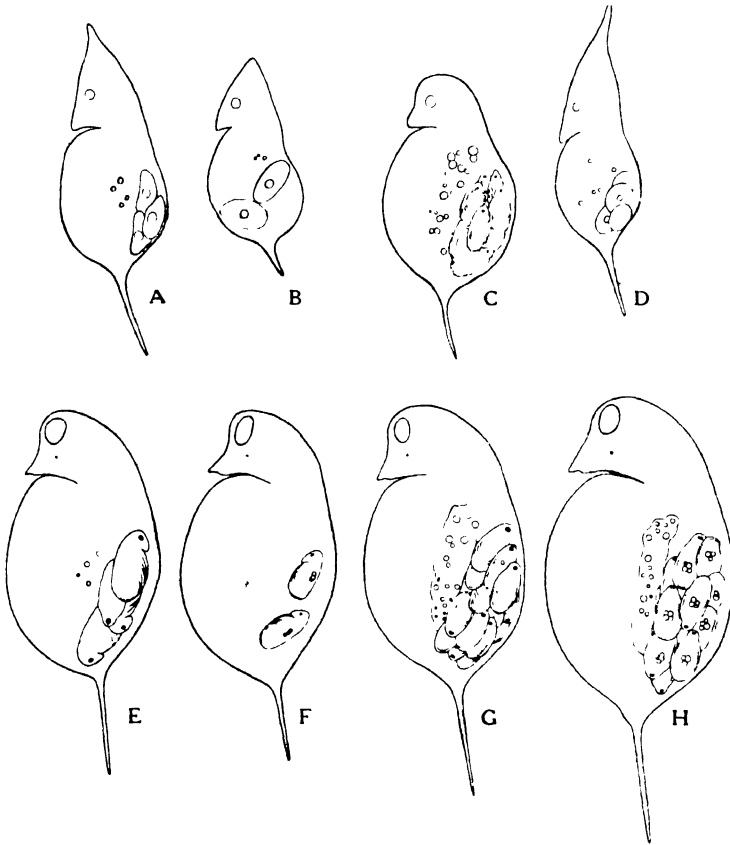


Fig. 5. A, B, Two parthenogenetic females of *D. cucullata* taken during the autumnal sexual period. They contain but few oil globules. $\times 26$. C, D, Two females of *D. cucullata* taken in the spring and summer respectively, at periods when the species is in vigorous parthenogenetic propagation. The parthenogenetic females then contain a large number of oil globules. $\times 26$. E, F, Two parthenogenetic females of *D. longispina* taken during the sexual period of the early summer. Such females contain very few oil globules, sometimes none at all. $\times 21$. G, H, Two females of *D. longispina* taken during the vigorous parthenogenesis of spring. A large number of oil globules are accumulated in the ovaries.

biological observations were made on the animals, showing whether they thrive well or the reverse, whether they are in full vigour or subject to depression. It has thus proved possible through certain of the experiments to point out a correlation between the modes of reproduction on the one hand and the degree of vitality of the animals on the other. In the following pages some such experiments will be

selected, and it will be pointed out how both earlier and more recent experiments support the depression hypothesis advanced above.

1. Papanicolau (1910 *a* and *b*) studied the reproduction of *Simocephalus vetulus* and of *Moina rectirostris* var. *Lilljeborgii*. The results in the case of both species were so similar that it is sufficient to mention the experiments with *Simocephalus vetulus*. Papanicolau kept this species in culture through twenty generations, each generation

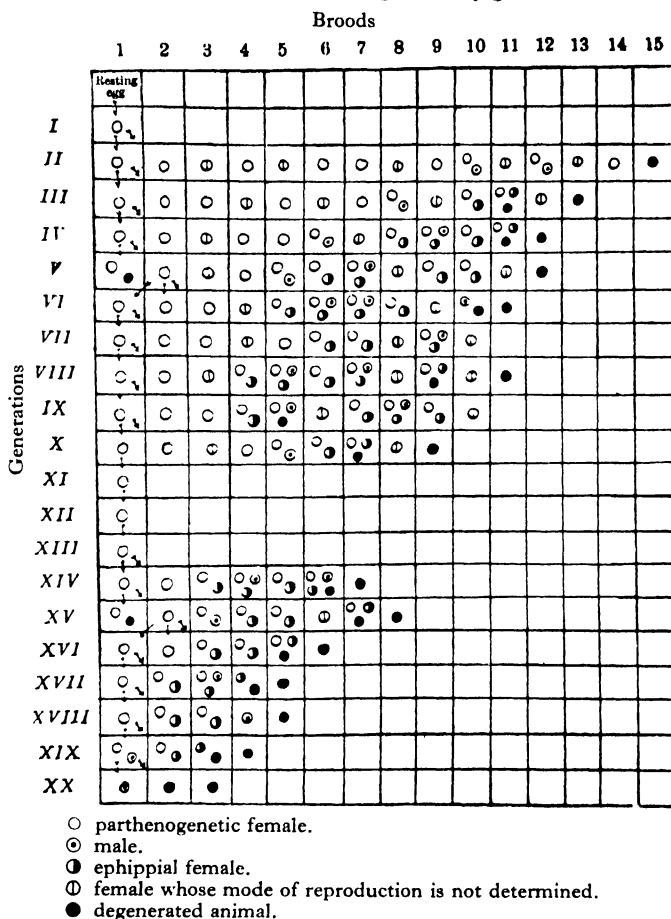


Fig. 6. Genealogical survey of a culture of *Simocephalus vetulus*. (After Papanicolau.)

comprising three to fifteen broods. A female hatched from an ephippium was chosen as ancestress. The animals were kept under so-called natural conditions: the temperature was 14–16°C., the water for the culture came from a pond, and the food consisted of triturated diatoms and green algae. The culture glasses held $\frac{1}{2}$ litre.

The results are shown in Fig. 6, which furnishes a general view of the sex of the animals and their reproduction in the various generations and broods. The figure shows also that in each generation males and ephippial females principally appear in

late broods, while as a rule the first broods of the generations are parthenogenetic females. But in the later generations, the parthenogenetic broods are few in number, males and ephippial females appearing almost at once. It is, moreover, especially worth noting that in (almost) every generation, simultaneously with or shortly after the appearance of the first gamogenetic individuals, degenerate individuals occur as well, while only in exceptional cases do such individuals appear in purely parthenogenetic broods. This interesting observation, then, presents very strong circumstantial evidence in favour of the hypothesis that gamogenesis is closely associated with a state of depression, while parthenogenesis is not. The state of depression is here so pronounced that it even manifests itself in the form of degenerate individuals. Even though the appearance of such animals may be supposed to be merely a culture phenomenon and is not probably of common occurrence in nature, still the very existence of such degenerate animals, and the fact that they appear simultaneously with the gamogenesis, is an interesting circumstance when looked at from

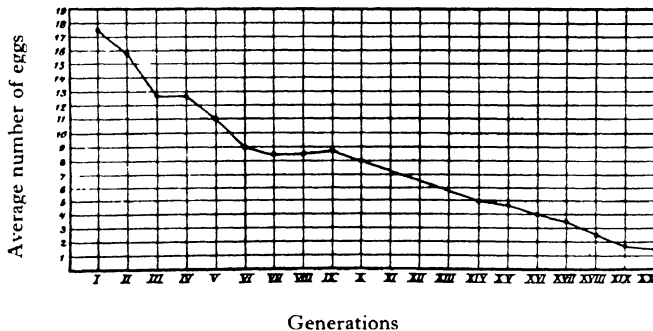


Fig. 7. Curve showing that there is a great decrease in the average number of eggs of *Simocephalus vetulus* from generation to generation in Papanicolau's cultures.

the point of view of the depression hypothesis. With reference to the degeneration of animals belonging to the last broods of the various generations, Papanicolau writes that it is an important fact that the animals of the last generation are extremely weak, often sterile and abnormal individuals, the majority of which die in their first stage of development, many already in the brood pouch of the mother animal, others soon after birth; only a few reach the age of puberty. Signs of degeneration may, however, also appear in a slighter degree in animals belonging to somewhat earlier broods.

2. The fact that in the experiments of Papanicolau (1910 *b*) the later generations contain fewer purely parthenogenetic broods than the first generations, shows that the depression advances from generation to generation. Papanicolau's statement regarding the average number of eggs (Fig. 7) confirms this observation. It will be seen that the number of eggs decreases in a marked degree from generation to generation. At the same time, as already hinted, the gamogenetic tendency increases at the expense of the parthenogenetic. This circumstance also agrees well with the depression hypothesis. As Fig. 7 relates to observations in the laboratory, it serves as

an excellent supplement to Figs. 3 and 4. These refer to observations in nature, where, it is true, the individual generations cannot be kept distinct, but where, nevertheless, it is possible to show a quite similar decrease in the average number of eggs before the sexual period.

3. Papanicolau (1910 *b*, p. 747) also examined the growth of *S. vetulus*, and determined, among other things, the average period elapsing between two moults in the various generations; the longer this period is, the slower, of course, is the rate of growth. The result of the investigations is shown in Fig. 8. It will be seen that the time elapsing between two successive moults increases very considerably from generation to generation. The slow rate of growth in the later generations, therefore, may also be regarded as the result of an increasing depression—which, as already stated, is accompanied by an increased gamogenetic tendency.

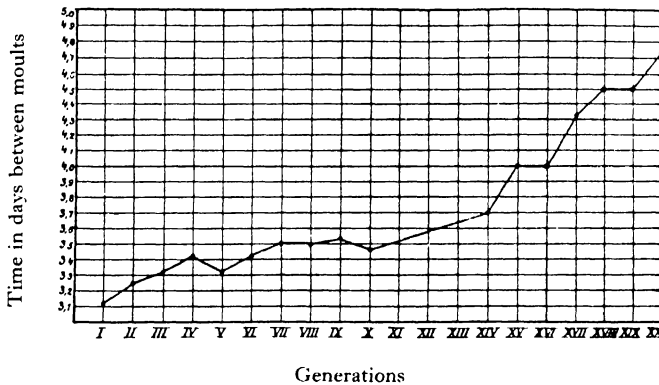


Fig. 8. Curve showing that the period between two successive moults is very considerably increased from generation to generation. (Experiments with *Simocephalus vetulus*, after Papanicolau.)

Analogous results have been obtained in calculating the rate of growth from brood to brood, and corresponding conclusions may be drawn therefrom: the mother animals gradually become weaker.

4. In regard to the growth of the ephippial females, Papanicolau writes (1910 *b*, p. 749) that it is slower and takes place by smaller steps than the growth of the parthenogenetic females. The average increase in size calculated from the first six moults in the ephippial females is only 0.136 mm., while in the parthenogenetic females it is 0.196 mm. It would seem, therefore, that the ephippial females are subject to a depression.

5. As regards the length of life of *S. vetulus*, Papanicolau (1910 *b*, p. 754) has found that parthenogenetic females from earlier generations and broods live longer than gamogenetic females. The parthenogenetic females may live for more than 2½ months, while ephippial females rarely live for more than 1½ months. Since the two classes of females had been living under similar conditions of culture, it is most reasonable to take the shorter length of life of the ephippial females as an indication that they are subject to a depression. The objection may perhaps be raised, however

that the duration of life is not a biological quality particularly well-suited to characterise the vitality of an organism; for it is quite conceivable that an organism in great vigour may, for that very reason, wear out all the more rapidly. (Compare MacArthur and Baillie, 1929, p. 253.)

6. Some abnormalities observed in *S. vetulus* were deformations of the forehead, carapace, antennae and legs. Most frequent, however, was a lack of pigmentation in the frontal eye. The abnormalities occurred most frequently in late generations and broods—that is to say, in those with the greatest gamogenetic tendency—as a result of a general enfeeblement in the animals.

Referring to the cause of some similar eye-abnormalities observed by Kapterew (1910), Papanicolau writes (1910*b*, p. 756) that only where the animals suffer from increasing weakness owing to a long period of parthenogenesis will alterations in the eyes occur among other signs of degeneration and that it is, therefore, impossible to regard such alterations as a result of external influences. We now know this explanation to be erroneous. Under suitable conditions, continued parthenogenesis is possible in the Cladocera to a far greater extent than Papanicolau knew; Banta (1917), for instance, kept *Simocephalus vetulus* in continual parthenogenetic reproduction through 130 generations, and Banta and Brown kept *S. exspinosus* through 384 generations (1923, p. 143). Furthermore, in their cultures of *Daphnia pulex*, Banta and Brown (1929, p. 81) had series of 767 parthenogenetic generations without diminution of vitality, and with other species they also obtained very large numbers. A similar result was obtained by van Herwerden, who from 1910 to 1916 kept up a parthenogenetic series of generations of *D. pulex*, uninterrupted by any case of gamogenesis; during that time this strain passed through about eighty generations and was perfectly sound and healthy (van Herwerden, 1920, p. 9). The eye-abnormalities which little by little appeared in the cultures of Papanicolau and Kapterew, must, therefore, have been caused by external influences, which gradually made themselves felt.

Continued parthenogenesis being thus, as stated above, possible through hundreds of generations without loss of strength, it is not possible to regard it as the cause of the biological phenomena—a diminution of the average number of eggs, and of the rate of growth, the appearance of degenerate individuals, of males and ephippial females, etc.—mentioned in the experiments of Papanicolau (points 1-5 above). These biological phenomena become more and more obvious from generation to generation and from brood to brood, that is to say, simultaneously with the continual parthenogenesis, but—for the said reason—not as a consequence thereof. The cause of the appearance of the phenomena is, therefore, to be sought in the gradual influence of the cultural conditions.

7. Ephippia of *D. cucullata*, which for more than 12 months had lain desiccated in a room, were exposed for about a month to a temperature of up to 25° C. by von Scharfenberg (1914, p. 32); then water was poured on them, and they were hatched. von Scharfenberg wanted to try whether this treatment would induce the production of males as early as in the first generation, as stated by Woltereck (1911, p. 125). In nature the males, as is well known, do not appear till much later

generations. v. Scharfenberg succeeded in making four of the females hatched from the said ephippia produce males, and one of them also produced an ephippium. He gives some information about two of these females. One, which produced only two males, looked "sehr kränklich" and perished after some time. The other, which produced in all five females and ten males, at a certain moment "sah sehr schlecht ernährt aus." These statements, then, would seem to indicate that the ex-ephippion females, at the time when they were producing males, were greatly depressed.

8. It was shown by Banta and Brown (1929) that the simple expedient of rearing a moderate number—ten—of parthenogenetic females of *Moina macrocopa*, crowded together in the same bottle, caused them to produce about 42 per cent. of male offspring, whereas uncrowded mothers produced only female offspring. It was also noticed in the course of the experiment that there was some relation between

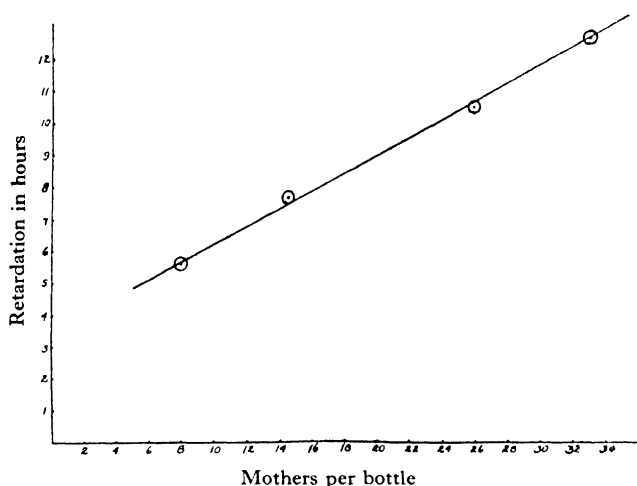


Fig. 9. Diagram showing the relation between the number of mothers reared in a bottle and the amount of retardation in the production of the first clutch of young. (After Banta and Brown.)

the time of release of a mother's first brood and the sex of the young in that brood. Since mothers reared ten in a bottle were slower in developing than their uncrowded sisters, as judged by the time of release of their first clutch of young, Banta and Brown thought that it would be of interest experimentally to increase the male percentage and to note the relation of this to the time of release of young by mothers so treated. The increased male percentages were induced by greater crowding of the mothers. The result was that the percentage of males produced was approximately proportional to the degree of crowding: two to four crowded mothers gave 4.9 per cent. of males, seven to nine crowded mothers gave 25.3 per cent. of males, twelve to seventeen crowded mothers gave 35.8 per cent. of males, twenty-three to twenty-nine crowded mothers gave 68.1 per cent. of males, and thirty to thirty-six crowded mothers gave 81.0 per cent. of males. At the same time as the male percentage rose in proportion to the degree of crowding, Banta and Brown also found a retardation

of the mothers' development, proportional to the degree of crowding; this latter condition is plainly shown in Fig. 9.

The experiment of Banta and Brown has, then, satisfactorily proved the production of males to be closely associated with a reduction in the rate of development of the mothers producing them—*i.e.* with a certain state of depression.

9. Banta and Brown (1929-30) have very clearly confirmed their result regarding the relation between the production of males and the rate of development of the mothers, having been able—not only by crowding but also in other ways—to increase and diminish the rate of development of the mothers. By means of ethyl alcohol (small doses), filtrates of dried adrenal cortex, thyroid, thymus, and muscle

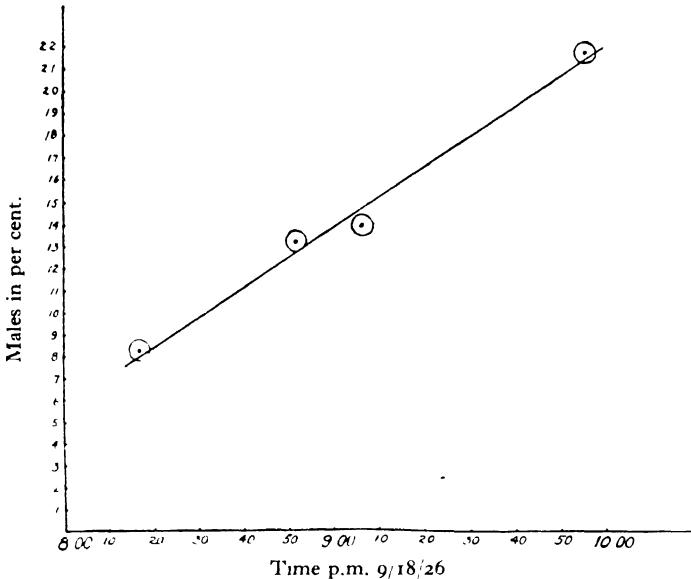


Fig. 10. Diagram showing the relation between the rate of development of the mothers and the percentage of males in their offspring. The time of release of the mothers' first brood is given as abscissae. Experiments with *Moina macrocopa*. (After Banta and Brown.)

tissue they succeeded in increasing the rate of development of *Moina macrocopa*. This increased rate of development largely counteracts the normally retarding effect of crowding the animals, and contributes to *reduce* materially the proportion of males produced by such mothers (Fig. 10). Under the influence of chlorethone, phenyl urethane or potassium cyanide, parthenogenetic females of *M. macrocopa* reached their reproductive age very slowly. Females thus *retarded* in their rate of development produce a much larger percentage of males among their first broods of young than untreated control females. The greater or smaller retardation in the development of the mothers and its connection with a greater or smaller production of males is in full agreement with the depression hypothesis¹.

¹ A. Tauson (1930, p. 105) tried the poisoning effect on *D. pulex* of various cations, more especially solutions of Na, K, Ca and Mg, mixed with the pond water in which the daphnids were kept in cultures. Tauson draws the conclusion from her experiments that none of these drugs are of any

10. Concerning the materials—chloretone, phenyl urethane and potassium cyanide—principally used by Banta and Brown to induce the production of males, the authors remark that such drugs are anaesthetics and that it is generally assumed that to a large extent anaesthetics inhibit the general metabolic processes of an organism. In support hereof they refer to investigations by Child (1910) and Allee (1912). Potassium cyanide in weak solutions was used to test the metabolic levels of animals (Child, 1913) or to reduce the metabolic rate of animals (Allee, 1912). The effect of both chloretone and potassium cyanide is supposed to resemble that produced by decreasing the supply of oxygen. Urethane belongs to the group of hypnotics and should produce somewhat similar effects to chloretone, opiates and other such drugs.

It is, therefore, probable that the said drugs, besides encouraging the production of males and hindering the growth of the mother animals, also to a large extent exercise a lowering influence on the general metabolic processes; this, then, presents further proof in support of the depression hypothesis.

11. Among Banta and Brown's many crowding experiments there are some (1929) in which the crowded mothers were subjected to treatments which neutralised the male-producing effect of the crowding method. Normally crowded bottles (ten mothers to 75 c.c. of culture medium) were, for instance, aerated in several ways and were found to produce a greatly reduced percentage of males as compared with their respective crowded control bottles. Thus in one experiment crowded mothers produced only 24.4 per cent. of males after the culture medium had been aerated by shaking every 3 or 4 hours for some time before the eggs were laid. The crowded control mothers, on the other hand, gave 37.9 per cent. of males. The experiment comprised altogether no less than 5115 young animals. Stuart, Cooper and Miller (1932) have discussed this experiment, their argument being that the shaking of a bottle containing crowded mothers may effect the medium in several ways. Two

importance in regard to sex determination. Na, K and Ca have a physiologically harmful effect in that they injure the vital functions and hinder the embryonic development. Mg is particularly poisonous, even in low concentrations. Furthermore, Tauson concludes (p. 110) that some of her experiments stand in contradiction to the above-mentioned results of Banta and Brown which showed a connection between a retardation in the development of the mothers and the production of males in their offspring. For Tauson was able quite clearly to prove a hindrance in the embryonic rate of development of the young, etc. without once finding males in these cultures.

The Tauson experiments are, however—as far as I can see—hardly able to shake the important result arrived at by Banta and Brown, since Tauson states that "die Versuche stets mit Weibchen begonnen wurden, bei welchen in der Zuchtkammer entweder Eier oder Embryonen auf frühen Entwicklungsstadien vorhanden waren" (p. 109), which means that in all probability the sex of the young animals to be developed from these eggs or embryos was determined long before. At any rate Banta and Brown (1929, p. 72) have shown that in *M. macrocopa* the sex is determined about 4 hours before the eggs are transferred to the brood pouch. But if the sex of the offspring mentioned in Tauson's experiments must be supposed to have been determined before the experiments were commenced, it is natural that no males appeared in the course of the experiments, in spite of the poison effect. For as far as can be judged from the tabulated results of the experiments, in by far the majority of cases the poison effect is so strong that the mother animal does not produce new broods in the course of the experimental period, but slowly brings into the world the young already to be found in the brood pouch.

In other tests made by Tauson the poison effect was weak, for instance in one culture a solution of 1/1000 mol. $MgCl_2$. In this case the embryonic development is checked at first, but soon the poison effect is overcome, the animals reproducing vigorously and forming a luxuriant culture, so that there is no reason to expect the production of males in this case either.

possibilities are at once apparent besides the eventual breaking down of excretory products. (1) Increase of food: it is a well-known fact that the aeration of a medium containing bacteria has a decided growth-stimulating effect on the bacteria. (2) Redistribution of food: within a few hours after a normal medium has been placed in a bottle there is considerable sedimentation of silt together with spontaneously agglutinated bacteria.

The said authors are also of opinion that, in addition, a third and even more important possibility must be considered, namely, that aeration or agitation of a crowded bottle affects the mothers and not the medium. Aeration or agitation may increase the activities of the mothers, which in turn will increase their metabolism, accelerating their development, with a subsequent increase in the number of female young. If this explanation of Banta and Brown's experiments is the right one or at any rate represents part of the truth—and this seems very likely—then this experiment is also in good agreement with the depression hypothesis.

12. Tauson (1930, p. 87) has examined the significance of various factors in regard to sex determination and development in *D. pulex*. Amongst other things the influence of temperature was studied. Several rows of culture glasses were arranged, each at a different temperature. The temperature varied from 0 to 30° C. Each culture glass contained 100 c.c. pond water mixed with foodstuff consisting of triturated algae (*Cladophora fracta*); into each glass were put 3–5 females of *D. pulex*, and the development of the culture was observed for up to 1½ months. Afterwards series which had lived at different temperatures could be compared.

The principal result was that temperature is an important factor both for the sex determination and for the development of *D. pulex*. A temperature of 15–25° C. is favourable to parthenogenesis; above 25° C. a hindering influence begins to make itself felt. At a reduction of the temperature to 14° C. males are produced, and at 12–14° C. females with ephippia. Temperatures below 15° C. are, therefore, significant for the determination of sex. Under such circumstances first males and, upon a further reduction of temperature, ephippial females will appear. Tauson is furthermore of opinion that the appearance of gamogenetic animals is due to unfavourable life conditions ("unter 15° C. erscheinen ungünstige Lebensbedingungen. . ." "Das Erscheinen von Männchen ist das erste Zeichen des Eintritts von ungünstigen Temperaturbedingungen" (1930, pp. 94–5). The supposition that the conditions of life are unfavourable in a cold culture (9–12° C.) in which ephippial females occur is further borne out by the fact that some of the young animals quickly die; others remain small, hardly grow at all, and gradually die off. In another similar experiment (10–12° C.) a number of the eggs degenerated in the brood pouch in the parthenogenetic females, and the latter remained sterile for a long time and then died, while at the same time other females started producing ephippia. Altogether, this agrees well with the depression hypothesis.

13. Tauson (1930, p. 98) also carried out experiments with *D. pulex* on the sex-determining significance of the quantity of nourishment. Part of the animals were daily supplied with abundant food consisting of squashed algae (*C. fracta*), others were insufficiently fed by having a little manure stirred up in water added to the

culture water. Other animals, again, had algae food for 24 hours, and after this time had elapsed they were placed for 48 hours in water purified by filtration through gauze No. 20. Then they again had algae food for 24 hours, and so on. These animals were starved to some extent. Finally, some daphnids were kept all the time in the filtered water, that is to say, in a severe starvation culture. The result of these feeding experiments proved to be the same as that of many other similar tests: nutrition plays an important part in the determination of sex. Insufficient nourishment entails first the appearance of males, later that of ephippial females. In dealing with the animals which have been exposed to severe hunger, Tauson also writes (1930, p. 103) that in addition to the phenomena already mentioned retardation of development and growth was observed; puberty occurred with some delay. Some animals did not attain maturity until the sixteenth day, while under normal conditions maturity is reached on the seventh or eighth day. Similarly only a few young are produced parthenogenetically by each mother animal, compared to what happens under normal feeding conditions. Thus Tauson's experiments show that at the same time as gamogenesis occurs, a state of depression has set in, manifesting itself in the slow growth of the animals, their belated arrival at sexual maturity and their slight parthenogenetic reproduction.

14. Finally, Tauson (1930, p. 115) has also examined the effect of the degree of acidity of the water on the determination of sex and arrives at the following result. If *D. pulex* is removed from water reacting almost neutrally to water which is slightly acid (pH 6.8) this change has no effect, but if the pH sinks to 6.7–6.3 the animals will produce males in fairly large quantities. If the pH sinks still further to 5.8, it has a fatal effect on the animals.

Alterations in the reaction in an alkaline direction produce a different result. Even a considerable increase in the pH of the culture water causes no production of males, only females are produced. Increase in pH also produces a physiological effect, it "wirkt stimulierend auf das Wachstum und die Entwicklung" (p. 124); this holds good for high pH values of up to 9.0. At still higher values, for instance pH 9.7, the increase in alkalinity continues to have a stimulating effect on the rate of growth and the development of the young, but it is fatal to the mother animals.

No information is available as to possible signs of depression in the animals in the cultures at pH 6.7–6.3 in which males were produced. But the animals must have been *relatively* depressed in comparison with those in the alkaline cultures. The statement that in the latter females alone were produced, and that a stimulated growth and development took place, is, furthermore, in perfect agreement with the depression hypothesis.

15. Certain daphnids—especially *Daphnia cucullata*—have, as is well known, a considerable seasonal variation, which chiefly shows itself in the summer by a considerable elongation of the head, the so-called helmet or crest, the animals being round-headed at other seasons. The appearance of the crest is dependent on favourable external conditions, above all on suitable temperature conditions (Ostwald, 1902). When, in the spring, the temperature of the water rises to about 14–16° C., the alteration takes place from round-headed animals to animals with high helmets

(Wesenberg-Lund, 1908, p. 189). The quantity of food is also of importance, plentiful nourishment promoting the formation of helmets, while scanty food hinders it (Woltereck, 1909, p. 123). The size of the helmet is, therefore, a good indicator of the vigour and vitality of the animals, even though internal factors may also be of some significance as far as the helmet is concerned.

With the above information in mind, it may be of interest to look once more at Fig. 1, p. 142. It shows a female *Daphnia* which was kept in a crowding culture in the month of July, that is to say, at a time when in nature the females have high helmets. In the species to which the depicted animal belongs the helmets grow so large at this season under normal conditions that the head becomes as high as the carapace (Fig. 5 D). The female from the crowding experiment (Fig. 1) thus has a much lower helmet than the animals living in the lake at the same time, which shows that it must be in a somewhat depressed state. Moreover, its reproduction is gamogenetic, contrary to what is the case with the females living in the lake, these being all parthenogenetic at that time.

As will easily be seen, the experiments described in this section all serve to support the depression hypothesis. Only by accepting this hypothesis will it be possible to understand how the very diverse sex-determining external factors can have the same effect.

IV. INVESTIGATIONS IN NATURE WHICH SUPPORT THE DEPRESSION HYPOTHESIS.

In the preceding section we have considered laboratory experiments proving the existence of a correlation between parthenogenesis and a high state of vitality in the daphnids, and between gamogenesis and phenomena of depression in these animals. The experiments therefore support the hypothesis that the transition from parthenogenesis to gamogenesis is caused by a depression induced by external conditions. In a similar way it is possible to adduce examples from investigations in nature showing exactly the same connection between the mode of reproduction (and sex determination) and the high or low vitality of the animals.

It is of importance to procure such corresponding testimonies from experiments and from observations in nature. Not only is the certainty of the results increased as a consequence of the larger number of mutually corresponding observations, but the two methods of research supplement each other, so as to enhance the significance or the value of the results. When the result of the experimental analysis agrees with the observations in nature, one may know that one has not been deluded by the experiments, that they are not mere artefacts and have not disclosed morbid phenomena in the organisms, or phenomena which are abnormal and so without interest. And, conversely, when the observations in nature are confirmed by laboratory experiments, these latter are a warrant that the contemporaneous biological phenomena observed in nature are not merely coincident by chance, but causally connected; in the laboratory the experimenter may repeat them at will and at any time he pleases.

We shall now mention some observations from nature:

1. One day in June Rammner (1932, p. 41) found that the surface of a pond near Quasnitz in the vicinity of Leipzig was stained red by great shoals of *Daphnia pulex*. The animals formed a wide red border along the shore; each of the rushes was surrounded from the surface of the water to a considerable depth by a thick red shoal of daphnids, and identical red conglomerations were to be found under every drifting leaf and every floating piece of wood. Such reddening of the water by *D. pulex* has already often been observed.

In this pond, over-populated with *D. pulex*, 26 per cent. of the animals were males, and of the full-grown females 21.4 per cent. were ephippial females; that is to say, then, that the intensity of the gamogenesis was considerable. At the same time Rammner noticed certain biological and morphological "Degenerationserscheinungen" in the animals. The parthenogenetic production of eggs was very considerably diminished as a consequence of the over-population. No less than 67 per cent. of the females had empty brood pouches, and only 11.7 per cent. of the sexually mature animals had quickly developing eggs. The very slight parthenogenesis was particularly clear from the fact that the females which had eggs at all, as a rule had only one or two; this was also the case with the large animals more than 2 mm. long. The maximum number of eggs found was seven. In this connection it is worth recalling the fact that under good conditions *D. pulex* may have a much greater number of eggs; in different populations an average of twenty to thirty-five eggs is not unusual. In addition Rammner's observations regarding the low number of eggs carried by the parthenogenetic females during the sexual period confirm certain facts which have already been mentioned (p. 142). About the morphological signs of weakness he writes that there were many animals whose carapace showed definite defects. The borders of the carapace had irregular dents or sometimes swellings; it looked as if small bits had been chipped off the carapace. Such irregularities could be found in one or in both halves of the carapace, and they occurred singly or severally (*vide* Fig. 11). Furthermore, the dorsal edge would not infrequently present one or more dents. The same deformities have previously been observed in daphnids which have been exposed to lengthy periods of starvation (Rammner, 1930). It is therefore to be supposed that the phenomena of degeneration already described were due to want of nourishment caused by over-population. A very small spine is even more frequent than defects of the carapace, and in many cases a spine is missing altogether (*vide* Fig. 11). Such animals are often very like the var. *obtusa*. While defects of the carapace are only to be found in full-grown females, the spine is also at times lacking in males, although this is rarely the case. In Rammner's opinion it is only to be expected that the males should react very slightly to unfavourable conditions, since, in a sense, they are reduced forms. Taken altogether the observations of Rammner have, then, furnished proof of an unmistakable coincidence of symptoms of depression and of gamogenetic reproduction.

2. It has been proved experimentally that when subjected to starvation daphnids lose weight with great rapidity before dying from hunger; Kerb (1910, p. 504), for instance, has shown that during a period of starvation the dry substance of daphnids

may fall to about a quarter of its original weight. The weight of daphnids being, as we have seen, a quantity apt to vary with external conditions such as the amount of nourishment, the idea readily presents itself that it may possibly be similarly influenced by other inhibiting external factors, and that, in other words, a low weight would be an expression of a state of depression in the animals. If this is correct, parthenogenetic females might be expected, according to the depression hypothesis, to have a higher average weight than gamogenetic females.

In order to try whether this supposition holds good, I determined the dry weight of parthenogenetic as well as gamogenetic females of *D. magna* and *D. pulex*. The determination, which has not yet been published, took place in the following manner. The animals were caught in the ponds in which they live in nature, and were at once measured, dried and weighed. As far as the results go, it can be stated that in

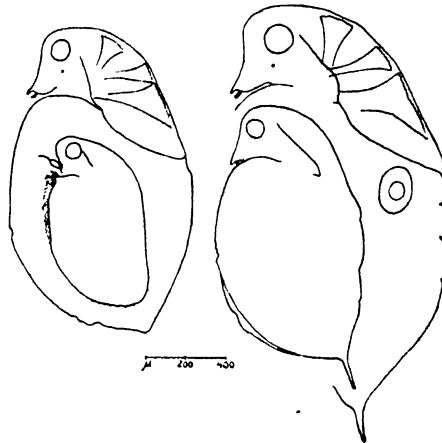


Fig. 11. The figure shows degenerate individuals of *D. pulex* from an over-populated pond. To the left, female and male devoid of spines; to the right, females whose dorsal and ventral contours show irregular indentations. (After W. Rammner.)

D. pulex the parthenogenetic females weighed 16.4–47.2 per cent. more than the gamogenetic females, and in *D. magna* 20.1–70.6 per cent. more than the gamogenetic. As an average for both species and for all the populations examined it was found that the parthenogenetic females weighed 38.1 per cent. more than the gamogenetic females—that is to say, that here again we have evident confirmation of the hypothesis that the latter are in a state of depression as compared with the former.

3. It has been mentioned that the growth of ephippial females in cultures may take place more slowly and by smaller steps than the growth of parthenogenetic females (p. 148). In perfect agreement herewith, it has also been found that in daphnids caught in nature, the average size of ephippial females may be less than the size of accompanying parthenogenetic females (Hartmann, 1915, p. 146; Berg, 1931, p. 66). This of course suggests that the ephippial females are in a state of depression. Furthermore, Hartmann remarks that the formation of winter eggs may make great demands on the organism, thus hindering the growth of the individual; if this is the case, it would further accentuate any previously existing depression.

4. Generally speaking it is correct to say that the seasonal variation of the Cladocera is most pronounced when the very best conditions prevail as regards temperature and nourishment; at such times, for instance, the crest is highest, whereas it is quite small at low temperatures and during hunger periods. Wagler (1912, p. 348) has also tried to prove the existence of a connection between seasonal variations and the cycle of reproduction. He chose for his investigation a *D. longispina* which had four sexual periods annually during the period of 5 years through which he kept it under observation. In one of those years he determined the ratio head-crest to carapace and the ratio head-crest to head-basis; at the same time he carefully observed the sexual periods. The result is shown in Fig. 12. When the first sexual period occurred at the end of May and the beginning of June, the animals

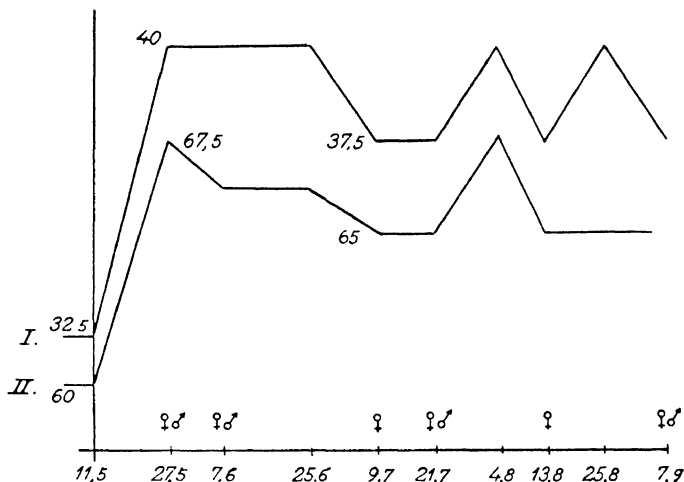


Fig. 12. *D. longispina*, Kospudner Pond. The dates of capture are given as abscissae. The male and female signs mark the dates when gamogenetic animals were found. The curves give the relation between height of head and length of carapace (I), and between height of head and base of head (II). As will be seen, the last three sexual periods occurred at times when the height of the head was small. (After E. Wagler, with slight alterations.)

had a fairly large crest; it was higher than that of the females which appeared in the early spring. As far as the crest was concerned, the population did not, therefore, show any sign of depression during the first sexual period, but in other respects it did. Wagler states that the parthenogenetic females at that time had a small number of eggs. The three ensuing sexual periods are seen to occur when the crest is low. He concludes, therefore, that there is a connection between the seasonal variation and the sexual cycles. This connection can be explained by means of the depression hypothesis, if we presume that the coincidence in time between the slight relative height of the crest and the sexual period is due to the circumstance that sexual periods occur during periods of depression. Wagler points out that these results from animals taken in nature agree very well with the experiments of Woltereck, in which daphnids suffering from a certain degree of depression showed a gamogenetic tendency and but slight indications of the capacity for variation.

The interesting connection, pointed out above, between the seasonal variation and the sexual cycles is, however, in need of confirmation, preferably by means of populations with a greater and therefore more easily discernible variation than the above-mentioned *D. longispina* of Wagler. In the course of such future investigations it will be desirable to try to distinguish between the variation and depression shown by the gamogenetic animals (and the contemporaneous parthenogenetic animals) and the reduced seasonal variation exhibited by the first generation after the ephippia; the former variation and depression are of considerable interest for the comprehension of the transition from parthenogenesis to gamogenesis. The latter variation—which Behning (1912, p. 57), for instance, considers important for the changes in the extremities of the Cladocera—is of no interest as far as the above problem is concerned, though it is important for the full understanding of seasonal variation.

Taken together, all the various above-mentioned observations must be said to support the depression hypothesis.

V. CYCLIC REPRODUCTION, SEX DETERMINATION AND DEPRESSION IN THE ROTIFERA: A COMPARISON WITH SIMILAR PHENOMENA IN THE CLADOCERA.

In this section the importance of external conditions in the reproduction of the Rotifera will be exemplified, and the question will be examined whether the connection of depression with the change from parthenogenesis to gamogenesis (and thus also with sex determination) which was found for the Cladocera may possibly exist in these animals too. A complete survey of the extensive literature on the propagation of the Rotifera is, indeed, precluded. But the importance of any possible generalisation renders it reasonable to attempt a comparison. In this way the problem is, at any rate, raised. Future investigators on sexuality in the Rotifera—and possibly also in other animal groups with a heterogonous reproduction, *e.g.* the Aphididae—may then take up the question for discussion and perhaps establish as a fact what can here only be suggested as a possibility or a probability.

The Rotifera constitute one of those groups of animals which most naturally invites comparison with the Cladocera. They live in the same localities, and both have a cyclic reproduction with an alternation of parthenogenesis and gamogenesis. Only a very few families among the Rotifera and some few forms of Cladocera form an exception to this rule.

The heterogonous reproduction of the Rotifera, however, does not quite resemble that of the Cladocera. The Rotifera have two kinds of females, amictic and mictic. In the amictic females reproduction is always by parthenogenesis—fertilisation having no influence—and they only produce females; these females may either be amictic, like their mothers, or mictic. The mictic females may also reproduce parthenogenetically, and in that case they only produce males; but they may also be impregnated, and in that case they produce resting eggs. Resting eggs are fertilised eggs.

Among the Rotifera there is a sharp distinction between the two kinds of

females, though as a rule they have the same appearance; among the Cladocera, on the other hand, the same female may produce both females and males (by parthenogenetic reproduction) and resting eggs (by gamogenetic reproduction). From the resting eggs of the Rotifera—as from those of the Cladocera—females are always hatched, and these females are always amictic. But already in the next generation mictic females may occur.

The appearance of the mictic females inaugurates the sexual period; whether the mictic females will produce males or resting eggs from which females will be hatched, will, as mentioned above, depend on whether fertilisation does or does not take place. Thus, in this way the actual sex determination is dependent on fertilisation. But whether the sexual period will occur at all depends on the factors which determine whether the amictic females will continue to produce amictic females or begin to produce mictic young. If such a change occurs in the reproduction of the amictic females, this determines the course of the reproductive cycle, and a necessary

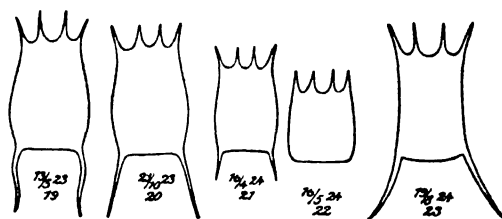


Fig. 13. *Anuraea aculeata*. The figure shows the seasonal variation in a Danish pond. During 1923 the species was a typical *A. aculeata* with two equally long posterior thorns (Nos. 19, 20); no sexual period was observed. In 1924, when the pond had become ice-free, the species occurred in a very small form with very short thorns (No. 21), and in May the thorns had totally disappeared (No. 22). Simultaneously a sexual period set in, and mictic females with chains of 3-4 male eggs or resting eggs appeared. When the sexual period was at an end, the maximum ceased and the rather few specimens which occurred during the rest of 1924 and the whole of 1925 had all well-developed posterior thorns (No. 23). Curiously enough, during the whole of this time no trace of a sexual period was seen; apparently all females were amictic. (After Wesenberg-Lund.)

precursor of the later sex determination has appeared. We shall now see whether, at this transition period in the reproductive cycle of the Rotifera, any sign can be found of a depression in the populations.

(a) From Wesenberg-Lund's extensive work on the periodicity and sexual periods of the Rotifera (1930, p. 125), the following observations on *Anuraea aculeata* may be quoted. During the spring and occasionally during the autumn large maxima of the species have been found. Random samples taken in different ponds and at different times show that it is in the spring that mictic females appear; even if maxima at other times of the year are rather large, a sexual period has very rarely been observed.

A. aculeata shows rather considerable seasonal variations; Wesenberg-Lund thinks that these variations are not only dependent on variation in external conditions, they also depend on an internal cycle, and are in some way connected with the sexuality and the development of mictic females. His investigations would seem to show that some of the most dominant forms of *A. aculeata*, such as *A. valga*, *A. brevispina*, and *A. curvicornis* (vide Fig. 13) almost always appear before and during

a sexual period. During this period the females have that peculiar appearance which makes us refer them to one of these seasonal forms. These seasonal forms may live for a shorter or longer period of the summer, but sometimes after the great spring maximum and the sexual period they disappear. During the summer the species is represented by specimens with well-developed posterior spines of equal length (*forma typica*, Fig. 13, No. 23), furthermore by forms which are on the whole larger than the spring forms; during the winter these forms with long spines almost invariably occur.

The large forms with well-developed posterior spines may perhaps have a sexual period and produce mictic females; Wesenberg-Lund, however, thinks that this is not the rule.

In some ponds the large forms, *forma typica*, will predominate for two or three years, no sexual period will occur, and none of the aberrant forms will appear. Sooner or later the irregular forms *A. valga*, *A. brevispina*, and *A. curvicornis* will then occur, and these forms are smaller than the main form, *forma typica*; they are less vigorously developed. If both posterior spines are present, they are small; in some ponds one of the posterior spines is wanting, in others both. Simultaneously with the appearance of these forms the sexual period begins; the mictic females belong to all the above-named forms. The maxima, the sexual periods, and the irregular forms are followed by minima, large forms with well-developed posterior spines, and no sexual periods.

Wesenberg-Lund is inclined to interpret the above-mentioned facts to the effect that "sexuality sets in in the life of the colony when its strength is spent" (1930, p. 131). The chief result of the sexual period is the resting egg, from which the colony begins a new life with larger and more abundantly equipped specimens. The period of degeneration causing the sexual period does not seem to occur in the history of the colony every year, often only every second year, or perhaps every third year. It frequently seems to be peculiar to a certain season, particularly the spring.

The sexual period may also occur in the autumn, or may set in at the beginning of the winter. If a colony is passing into a period of degeneration, and this happens when the ice covers the ponds, the rule is that *A. aculeata* is present as *forma typica*, but in rather small specimens. Shortly after the ice has disappeared, at a temperature of 14–16° C., the degenerate forms occur, whereupon a sexual period ensues.

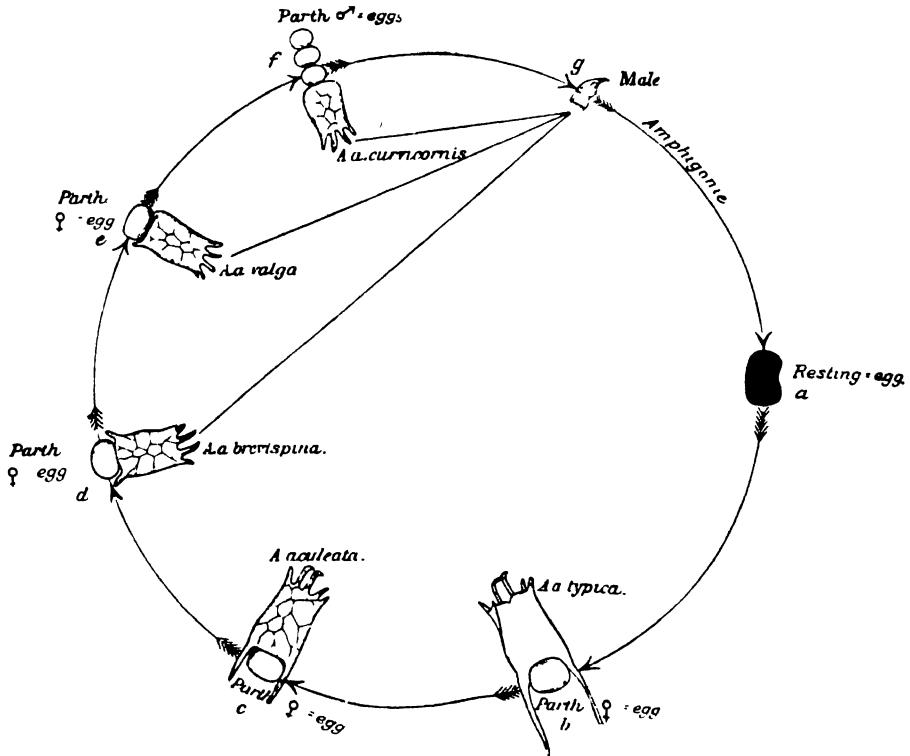
Another interesting fact of sexual biology is worth noting. As a plankton organism in larger lakes *A. aculeata* lacks all those forms which Wesenberg-Lund regards as degenerate. At the same time, in the pelagic region of larger lakes, no sexual period and no mictic females have ever been found, as far as we know. Apparently only amictic females occur there. Variations in size, local as well as seasonal, may be found, but the numerous pond forms have never been found as participants in the pelagic life of larger lakes. These observations, as well as those mentioned above, corroborate "the fact that there is a connection between sexuality and the degenerate forms..." (Wesenberg-Lund, 1930, p. 131). It is quite obvious, too, that the observations are in good agreement with the facts which led to the advance-

ment of the depression hypothesis for the Cladocera. It should be added, however, that Wesenberg-Lund is inclined to think that internal factors, *e.g.* the distance from a sexual period, are partly responsible for the appearance of the degenerate forms. External conditions, too, especially temperature, viscosity, and nutrition, all influence the form of the organism, *e.g.* the curvature and thickness of the posterior spines; they may likewise influence the size and varying degree of development of the spines, but according to Wesenberg-Lund, these factors are also dependent on the length of the parthenogenetic period.

(b) The above-described connection between the cyclomorphosis and the reproductive cycle of *A. aculeata* has also been demonstrated by laboratory experiments carried out by Krätschmar (1908, p. 623). Keeping *A. aculeata* in cultures, he made changes in the temperature, lighting, nutrition, etc. Under all conditions reduction series occurred. Every parthenogenetically produced individual had undergone an easily observable, rather considerable reduction in relation to its mother individual; the size of the body as well as the length of the spines had been reduced. Thus individuals with long spines produced only individuals with somewhat shorter spines; mother animals with short spines exclusively produced progeny with two short spines, a single short spine, or no spines at all. When a certain minimum had been attained, some of the individuals with short spines or without spines produced male eggs and—if fertilisation took place—resting eggs. A diagrammatic survey of the experimental results is shown in Fig. 14, from which it will appear that there is a connection between the sexual cycle and the changes of form in the various generations. Each form of the species has its specific scientific name. In the large and long-spined forms reproduction is by parthenogenesis. Gamogenesis occurs in the reduced—small and short-spined—forms. These are all observations which accord with the depression hypothesis.

Among the causes which may possibly have brought about the reduction, Krätschmar mentions a successive diminution of the vitality of the parthenogenetic females, in a way a "Degenerationsvorgang." He supposes that reproduction by parthenogenesis may occur in a rotifer species through a certain number of generations, when a certain exhaustion will supervene ("senile degeneration") and so a need for gamogenetic fertilisation. It must be noted, however, that the facts no longer would seem to warrant the general assumption that long-continued parthenogenesis is not possible without producing degeneration. In the course of time too much evidence of the reverse has come to hand (*cf.* Vandel, 1931, p. 318). In cultures of Rotifera parthenogenesis has been allowed to continue for a very long time. Whitney (1912) carried a series of *Hydatina senta* through 384 parthenogenetic generations and another series through 503 parthenogenetic generations; in nature, however, *H. senta* produces only 6–30 parthenogenetic generations (Wesenberg-Lund, 1930, p. 107). In Whitney's experiment with *H. senta*, at the 75th parthenogenetic generation there was no noticeable decrease of vigour; but much later this gradually appeared as the generations increased and the colony became older. It then turned out that the rate of reproduction had decreased very decidedly. Whitney, however, quite rightly thinks that this decrease in the rate of

As a matter of fact, Krätschmar thinks it possible that conditions other than senile degeneration may contribute to the occurrence of gamogenesis. In this connection it is worth recalling that even if the external agents (temperature,



nutrition, etc.) usually examined in these experiments have no effect, environmental conditions may nevertheless be determining factors. For the experiments may have been carried out under circumstances which differ so much in other respects from the conditions under which the experimental animals live in nature that the negative results arrived at are not convincing. Further, it is possible that external factors other than those examined are of importance; thus there can no longer be any doubt that crowding factors—and thus in a way the number of the animals—play a part in

the reproductive cycle of the Cladocera; but earlier investigators, being ignorant of this fact, could not take account of it.

In addition to his laboratory experiments, Krätschmar has also been able to show that in *Anuraea aculeata* in Lunz Obersee gamogenetic resting eggs only occur in reduced forms with a short spine.

(c) With regard to *A. cochlearis* Wesenberg-Lund (1930, p. 133) has made observations in a single locality, of quite the same nature as the investigations on *A. aculeata* mentioned above. *A. cochlearis* in its typical form has one long backward-directed spine; in *forma tecta* the spine is quite reduced. Between these two extremes there are intermediate forms with more or less reduced spines, but these forms only occurred in a few of the localities investigated by Wesenberg-Lund. In one of the ponds examined, however, there occurred both *forma typica*, *forma tecta*, and intermediate forms with somewhat reduced spines. In this locality the typical forms may predominate for several years; occasionally the species may be very similar to the *forma tecta*, and in some years it may be reduced to this form. The sexual periods are always connected with the reduction period.

(d) Tauson (1925, 1927) has examined experimentally the dependence of sex determination on the chemical composition of the water. The experimental animal was *Asplanchna intermedia*. Three to ten mature amictic females were placed in glass vessels containing water of various chemical compositions. The collective experiments gave the following results. The transference of rotifers from one medium, for instance pond water, bog water, river water, to another differing in chemical composition from that in which the rotifers had hitherto lived, resulted in the production of mictic females, and so also in the production of males. The percentage of mictic females decreased gradually as the animals accustomed themselves to the new medium. When a complete adaptation to the new medium had taken place, the mictic females entirely disappeared. The occurrence of mictic females thus proved to be a reaction to the change in the chemical composition of the medium. But the experiment showed yet another thing which is of special interest to us. On the first day after the transference of the animals from one medium to another, no increase in the number of individuals was ever observed. The change of medium "hat einen hemmenden Einfluss auf das Tempo der Entwicklung." The number of females was not increased the next day; not until the third or fourth day did mictic females occur—in connection with the decreased rate of development (Tauson, 1925, p. 293).

(e) Tauson also has experiments on the sex-determining influence of nutrition (1927, p. 353). No influence from a qualitative change in the nourishment could be demonstrated, but an effect was seen from a quantitative change of the food. The experiment was made as follows. A number of amictic females of *Asplanchna intermedia* were starved for 3 days. Then five such starving females were fed abundantly with the same food—*Paramecium* culture—with which they had been fed before the beginning of the experiment. They then at once proceeded to produce mictic females. Thus the beginning essential to a sexual period had been made. Simultaneously the mother animals that had been exposed to 3 days' starvation showed several

symptoms of exhaustion: they were inert and quite transparent, like shadows; their stomachs shrank and changed to a lighter hue; instead of the previous brown colour they turned a light yellow. The ovary grew smaller because the yolk-mass was absorbed by the females, a phenomenon generally observed with strong hunger. Tauson thinks that the diminished mass of yolk in the ovary of the starving animals resulted in the formation of smaller eggs, from which then, the mictic females developed (from which, again, the males developed).

(f) The literature contains many statements showing that the sexual periods of the Rotifera—and of the Cladocera—occur at times when the species in question is extremely common; it is then generally said to have its maximum. The maximum is produced by a very vigorous parthenogenetic propagation. The species, then, has proved very vigorous in that respect, and there is a temptation to believe that both the maximum and the sexual period connected therewith are the outcome of a high degree of vitality in the species (see Shull, 1925, p. 144). This, however, probably applies only to the maximum, not to the sexual period. For it is known from the study of the maxima and sexual periods of the Cladocera that the sexual periods by no means set in simultaneously with the maxima, but not until the culmination of the maxima has been attained, and so they have for some time been preceded by a vigorous but pure parthenogenesis (see *e.g.* Berg, 1931, p. 186). This vigorous, pure parthenogenesis results in a great number of individuals, and thus in conditions of depression similar to those found in the crowding cultures—and not until then does the change to gamogenesis take place.

The method of crowding in order to produce gamogenesis in various species is, therefore, one that very closely imitates what takes place under similar conditions in nature. Hence it would be of considerable interest to ascertain the nature of the factors that cause crowding to influence the mode of reproduction, as it is reasonable to suppose that the results arrived at would be similar to those obtaining in nature.

In the Rotifera, the connection between the maximum and the sexual period would seem to be of a similar kind to that indicated for the Cladocera. Here, too, the sexual period does not set in simultaneously with the maximum, but not until somewhat later. For numerous rotifer species it has been seen that, while the parthenogenetic generations immediately preceding a normal sexual period are extraordinarily productive and fill the waters with myriads of individuals, the males do not appear until the productivity has reached its maximum (Wesenberg-Lund, 1898, p. 202). When the above-cited author saw that a species of rotifer appeared as the principal form in a lake, he knew that the sexual period “bald eintreten würde.” Similar observations have later been made by other investigators, and Wesenberg-Lund’s continued studies (1923, p. 198) have corroborated them. He also states that the length of the maxima may vary from one to several weeks; it may also happen that they suddenly cease because of the setting in of a sexual period. Thus it will appear from this that in the Rotifera as well as in the Cladocera there is first a maximum and then, following immediately upon this, a sexual period. Hence with regard to the Rotifera, too, the supposition would seem to be warranted that the maximum is a

link in the causal chain which alters the mode of propagation; possibly the maximum produces a state of depression among the animals¹.

The above does not pretend to be a complete survey of the propagation investigations on the Rotifera; such summaries have lately been given by Tauson (1925, p. 130), Luntz (1926, p. 268), Shull (1929, p. 218), and especially by Wesenberg-Lund (1930, p. 6). But attention has been called to the above-cited experiments and investigations, in order to show that there is a good deal which would seem to indicate that in the Rotifera the transition from parthenogenesis to gamogenesis (and so also the sex determination associated therewith) is caused by a state of depression, and that this state of depression may be produced by the influence of various external agents.

It must be admitted that this conjecture is not supported by those experiments and investigations from which the investigators would infer that the reproductive cycle of the Rotifera is determined by internal, hereditary factors. On the other hand, the authors who attribute decisive influence on the mode of reproduction to external agents have statements very similar to the above-mentioned hypothesis. Thus Luntz (1929, p. 208) writes that the mechanism through which external factors cause a production of males is unknown but apparently there is a summation of, perhaps, harmful influences.

There is a temptation to draw further comparisons with other groups of heterogonous animals, for instance, the Aphididae. In these animals, too, there is an alternation between parthenogenesis and gamogenesis, and as for the Cladocera, a long series of papers have been published, dealing with the influence of external factors on the cycle (see *e.g.* Shull, 1929, p. 218). These plainly show that external conditions, at any rate among many species, are able to exert a far-reaching influence on the mode of propagation. On the other hand, as far as I can see, the literature does not seem to mention signs of depression in the populations simultaneously with the setting in of gamogenesis, not even when this is evidently connected with the influence of unfavourable external conditions. This may of course be due to the fact that the signs of depression have been overlooked; certain information would seem to suggest, for instance, that there is a reduction in size of the individuals in populations where gamogenesis sets in. An investigation of this question would be interesting. Possibilities for a fruitful comparison might perhaps also be found among other animals, for instance, *Miastor*, a paedogenetic dipteran in which crowding conditions have been found to exert an influence highly reminiscent of the observations on the Cladocera (Harris, 1923, p. 407; 1925, p. 89).

On the other hand, caution should be exercised in generalising; it should be remembered that parthenogenesis has arisen in so many ways from gamogenesis that Vandel is no doubt justified in writing "c'est la raison pour laquelle il n'est pas permis de généraliser en parthénogénèse et d'appliquer les conclusions tirées d'un

¹ For the Rotifera it has not been possible to obtain experimental corroboration of the connection between the maximum and the sexual period; on the contrary, Whitney found (1929, p. 269) that in crowded cultures of *Hydatina senta* the production of males is markedly reduced. How this disagreement between the laboratory experiments and the observations in nature should be explained is for the present unknown.

cas bien étudié aux autres cas de parthenogénèse" (1931, p. 310). Only the presence of similar observations—as in the case of the Cladocera and the Rotifera—warrants general conclusions.

VI. REMARKS ON PECULIARITIES CONNECTED WITH THE STATE OF DEPRESSION.

As signs or indications of the depression which is closely associated with the transition from parthenogenesis to gamogenesis in the Cladocera and the Rotifera, various characters and vital functions of the organisms concerned have been used. In the following paragraphs we shall try to characterise this state of depression more closely.

It has been plainly shown that gamogenesis is associated with certain signs of depression, whereas parthenogenesis takes place when the animals thrive well: numerous examples have proved the connection. It must not be expected, however, that no exceptions can be found. The possibility of finding such exceptions lies in the fact alone that the state of depression is not a sharply defined state in which all functions are reduced to a certain degree. It is quite possible that only the greater part of the functions are reduced, but others not. A certain external influence may very well be conceived to depress some functions and stimulate others. Hence the depression of some functions will perhaps not always be associated with gamogenesis.

An influence which causes depression of certain functions and stimulation of others in a cladoceran female, and which is anti-gamogenetic, is exemplified in some experiments with hot cultures carried out by Papanicolau (1910 *b*, p. 762). Papanicolau kept *Simocephalus* and *Moina* in cultures at 22–24° C. and compared these hot cultures with cultures at room temperature. The heat had a depressive effect on the animals; it reduced the average number of eggs and the number of broods in the parthenogenetic females and caused a degeneration of the animals after comparatively few generations. Finally it caused a reduction in the size of the eggs and in the size of the new-born animals and the animals in the later stages of their development. On the other hand, the heat stimulated the rate of development, reducing the average time between successive broods. And the heat did not serve to further gamogenesis, as might perhaps have been expected after the many signs of depression; on the contrary, it delayed the incidence of gamogenesis and reduced its intensity. Thus one of the hot cultures gave 24.3 per cent. of gamogenetic animals, while the culture at room temperature gave 68.6 per cent. So despite its depressive effect in several respects, the increased temperature did not cause increased gamogenesis, but had an anti-gamogenetic influence. This specific effect, however, does not render invalid the main depression hypothesis; and, as a matter of fact, it is seen that the increased temperature did not have an exclusively depressive effect, but it stimulated some functions, *e.g.* the rate of growth.

Other observations of depression phenomena not accompanied by gamogenesis can probably be explained in a similar way (see Gajl, 1927, p. 936); or they may possibly be due to the fact that the depression has not attained a suitable magnitude.

It has been mentioned (p. 150) that Banta and Brown's experiments support the depression hypothesis. To them, too, the idea of depression as a sex-determining factor is hardly foreign. At any rate, they designate the drugs which they use to produce a retardation in the development of mothers, and so a production of males, "depressing agents" (1930, p. 49). But they consider it possible that the retardation itself is the causative factor in male production. This they express by writing amongst other things that "the production of males is closely associated with, or caused by, a reduction in the rate of development of the mothers producing them" (1929, p. 318). The idea of a state of depression as the decisive factor also seems familiar to them, since they write (1930, p. 49) of the sex-determining drugs that "it is generally assumed that anaesthetics inhibit to a large extent the general metabolic processes of an organism."

It was mentioned above (p. 164) that Tauson, in her investigations on the Rotifera, found signs of depression in the females that produce the mictic females, that is to say, at the transition to the sexual period. But the reverse is not always the case. If depression is produced through certain external influences, it does not necessarily inaugurate a sexual period. This is shown for instance by investigations on the effect of the calcium ion. The calcium ion, which is added in the form of calcium carbonate, chloride or sulphate to alkaline pond water in which cultures of *Asplanchna intermedia* are living, very considerably paralyses development and growth. In such cultures a very slight increase in the number of individuals is seen, whereas there is a rapid increase in cultures without the calcium ion (Tauson, 1925, p. 381). Nevertheless, the calcium ion has not the effect of increasing the production of mictic females (Tauson, 1925, p. 313), but like certain other ions it has no effect in that respect.

The conclusion would seem to be inevitable that the depression may be limited to certain functions, at any rate in these animals. The state of depression has only interfered with certain functions—growth, etc.—but has not changed the mode of propagation.

Relative nuclear volume, reproduction, and depression. Under normal conditions, the ratio obtained when dividing nuclear volume by cytoplasmic volume (the Kernplasmarelation, the relative nuclear volume K/P) has a certain value (the Kernplasmanorm). Any disturbance in this ratio due to a change in either nuclear or cytoplasmic volume is presumed to lead to a tension (Kernplasmaspannung) which is assumed to be a factor of great importance in the physiology of the cells.

As is well known, Hertwig found (1903, p. 49) that the relative size of the nucleus in the Protozoa varies in relation to their life cycle. Continued division of an animal leads to an increase of K/P . After his first investigations it was tried whether this idea could also be applied to biological conditions in the Metazoa.

What is of special interest to us here is the possible connection of K/P with periods of depression in the Metazoa, with parthenogenesis, and with the change from parthenogenesis to gamogenesis.

In Protozoa in which periods of depression were common Popoff (1907, p. 59) found that the state of depression was accompanied by an increase in the size of the

nuclei. If the Protozoa were exposed to certain chemical influences, both depression and nuclear increase occurred (Popoff, 1909). The surmised connection between depression and K/P is also considered probable by Hertwig (1908, p. 1). In the Cladocera Hartmann (1919, p. 58) thought he found a high K/P ratio in periods of depression.

Popoff (1907, p. 73) infers that protracted parthenogenesis results in an increase of the Kernplasmarelation, and Issakowitsch (1907, p. 240) assumes the same for the Cladocera. But Shull (1922, p. 283), who has studied the nuclear volume and life cycle of a rotifer, *Hydatina*, thinks that it is too early to generalise with regard to the effect of parthenogenesis on the nuclear volume. The main result emerging from his careful experiments is that there is no change in the relative nuclear volume in relation to the mode of propagation.

Certain degeneration phenomena among the Cladocera makes Issakowitsch infer (1907, p. 240) that the transition from parthenogenesis to gamogenesis is controlled by changes in the Kernplasmarelation. Papanicolau (1910 *b*, p. 796) has made a brief histological investigation of the intestine of the Cladocera and draws the conclusion that the transition from parthenogenesis to gamogenesis is accompanied by an increase in nuclear volume. He appends some figures to his exposition but admits that the investigation is insufficient and not completely convincing. Hartmann's more comprehensive and thorough work leads him to the same assumption (1919, p. 60). Strohl (1908, p. 821) argues against this in favour of the reverse, and Woltereck (1911, p. 107) discusses the problem and arrives at the result that changes in the Kernplasmarelation cannot be the real cause of changes in the mode of propagation though they may possibly be an effect of the factors which determine the course of the propagation.

In the opinion of Hartmann it has been conclusively shown that in August, when the gamogenetic propagation in the cladoceran *Bosmina* has attained its maximum, the Kernplasmarelation and certain other cytological relations are conspicuously large. He considers these changes to be certainly and closely associated with the gamogenetic propagation, and thinks it probable that an internal factor—a state of depression produced by the high number of individuals in the generation—in conjunction with external factors (a high temperature, metabolic products, the lack of nourishment, etc.) induces the gamogenesis. According to Hartmann, protracted parthenogenesis is also supposed to cause an increase in the Kernplasmarelation. Hartmann's work is very comprehensive and considers many possibilities; there can be no doubt that he has been able to demonstrate changes in the Kernplasmarelation which are connected with the seasonal variation of external factors (temperature, etc.). On the other hand, his work lacks conclusive evidence of a connection between the cytological changes demonstrated by him and the change in the mode of propagation from parthenogenesis to gamogenesis (cf. Shull, 1922, p. 313). The same applies to the effects of a protracted parthenogenesis which, as mentioned above, is also, according to Hartmann, supposed to produce an increase in the Kernplasmarelation.

Even though the connection between the changes in the Kernplasmarelation

and in the mode of propagation cannot be said to be conclusively documented, it is nevertheless of interest to consider some of the inferences which Hartmann deduces from his investigations on the Cladocera (1919, p. 80): "Metabolic products, too rapid a rise in temperature and insolation, as well as lack of nourishment may bring about a state of depression; this often manifests itself in the incidence of a sexual period, which, it is true, is not on that account to be regarded as caused by purely external factors. The cytological symptoms in the cells of the intestine in such a state of depression, caused by external factors and the sexual period, are an increase in the Kernplasmarelation, in the nuclear relation, and in slight degree in the relative size of the cells. The growth of the body and consequently the growth of the cells is inhibited. . . . This depression in connection with a sexual period may again, when the external factors become more favourable, be made to regress considerably—though never quite; the same is the case with the cytological conditions." Hence, as will appear, Hartmann's view is in the main in good agreement with the other arguments that support the depression hypothesis. Whether his results are sufficiently well founded is a different question.

It is necessary to view with reservation the importance Hartmann attaches to internal factors; he writes that in the course of the generations, quite independently of external factors, an ageing, a depression of the cycle, sets in as a consequence of the purely parthenogenetic succession of generations, and this lasts until the colony dies off. The cytological and physiological characters of this latter depression are identical with those of the transient sexual depression released by external factors. Here, however, it must be noted that it can no longer be regarded as correct that long-continued parthenogenesis is able to produce a state of depression, etc. in these animals. For it has proved possible to make Cladocera reproduce parthenogenetically in cultures through numerous generations without any gamogenetic individuals being observed, and without any symptoms of decreased vigour or loss of vitality in the animals. Experiments illustrating this important observation have previously been mentioned (p. 149). To these must be added the more or less conclusive cases of protracted parthenogenesis observed in nature; as examples may be mentioned *Daphnia longispina* in the Vierwaldstättersee, in which no sexual period has ever been observed (Burckhardt, 1900, p. 265; Berg, 1931, p. 147). Further, it has been shown that parthenogenetic individuals of *D. cucullata* which have wintered, still retain their ability to pass into strong parthenogenetic reproduction, though they are removed by a long series of parthenogenetic generations from the last resting egg (Berg, 1931, p. 125). Continued parthenogenesis, therefore, must be regarded as without any depressive effect.

Even though the above-mentioned changes in the Kernplasmarelation are coincident with the transition from parthenogenesis to gamogenesis and—provided they are reliable—would seem to indicate a state of depression at this transition, this does not render acceptable the doctrine of the *K/P* relation derived from Hertwig. For this would presuppose that parthenogenesis in itself had a certain harmful effect which was gradually manifested cytologically. But as has just been shown, parthenogenesis does not necessarily cause a depression. Hence, when previous

authors refer to a state of depression in connection with Hertwig's doctrine of the Kernplasma, something different is meant from what has been mentioned in the present work. The reference was then to a lack of cytological equilibrium, caused in females of late generations by protracted parthenogenesis. The state of depression which we have described in the Cladocera, however, is due to the influence of external factors, and may be evoked even in early generations.

Even if the state of depression has not been produced by long-continued parthenogenesis and has no connection with the number of the generation, but is a state of weakness occurring in certain individuals, it may nevertheless in a way be said to be an internal cause of the change in the mode of propagation. The state of depression of course is an "internal" state; further, it is quite conceivable that the cumulative effect of external, only slightly depressive causes, active through several generations, may finally produce a change in the mode of propagation of certain generations. (As regards the transference of a gamogenetic tendency from one generation to another, see Berg, 1931, p. 143.) But the "internal" state of weakness which thus conditions the gamogenetic propagation, is not genotypically associated with the generations in question; as stated above, under favourable external conditions it may be changed, and parthenogenesis may be continued through hundreds of generations. And even if parthenogenesis is continued through numerous generations, the gamogenetic tendency in the late generations is still the same as in the early generations, so long as the external conditions are the same (Banta, 1925, p. 50).

Issakowitsch (1907, pp. 226, 235) has shown that nutrition and temperature are sex-determining factors in the Cladocera, a low temperature and starvation causing production of males and ephippia. In some of his cultures he induced a state of depression in the animals at which they just oscillated between a production of ephippia and a parthenogenetic production of females. It is interesting to note how this drop into or rise from a state of depression can be brought about. After an unimpregnated female of *Simocephalus vetulus* has cast off an empty ephippium, the next brood, even at low temperatures, consists of parthenogenetic females only; if the low temperature had any immediate influence, gamogenetic individuals might have been expected. Now, however, according to Issakowitsch, we must imagine the following process to take place in the female. The unfertilised resting egg is dissolved in the ovary and resorbed, which means that the ovary of the animal receives nourishment in certain quantity. There is therefore reason to suppose that this nourishment is the cause of the succeeding broods of females. After their birth the previous relation between nutrition and temperature is re-established, and the next brood, if the temperature is still low, will consist of gamogenetic animals. The fact worth noting in Issakowitsch's observations is that under certain conditions so comparatively slight an additional supply of nourishment as the resorption of an unfertilised resting egg may cause a discontinuance of the depression.

From what has been stated above we can make the following hypothetical statements regarding the state of depression which is associated with the change from parthenogenesis to gamogenesis:

1. The state of depression is not a sharply defined state in which all the functions

of the organism are reduced by a certain quantity; it is conceivable that only the main part of the vital functions are reduced, while others are not.

2. The depression may possibly be induced by factors which to a certain extent inhibit the general metabolic processes of the organism.

3. Even though the change from parthenogenesis to gamogenesis is associated with phenomena of depression in a species, a certain degree of depression may very well be produced, in some cases without changing the mode of propagation. It then affects other functions only.

4. It is possible that the depression may manifest itself cytologically by an increase of the Kernplasmarelation, etc., but these cytological symptoms of depression cannot be regarded as a consequence of parthenogenesis continued through numerous generations, since long-continued parthenogenesis must be considered to be without any depressive effect. The state of depression dealt with in this article is due to environmental factors and may be induced even in early generations.

5. Unfertilised resting eggs have been observed to be resorbed in *Simocephalus vetulus*. Such a comparatively slight "supply" of nourishment as the resorption of a resting egg may under certain conditions cause a discontinuance of the state of depression for a time, and induce the production of parthenogenetic females.

It has been shown that many entirely diverse factors—temperature, nutrition, calcium-ion concentration, other chemical agents, crowding factors, etc.—may cause depression. Depression causes a transition from parthenogenesis to gamogenesis, and since this is the case, it is understandable that such extremely diverse factors can have the same influence on propagation and thus on sex determination. Banta and Brown (1929, p. 74) have shown very clearly that the critical time for sex determination is about 4 hours before the parthenogenetic egg is transferred to the brood pouch (in *Moina macrocopa*); before that time the sex may still be influenced, but not after that time. The maturation division probably takes place about $1\frac{1}{2}$ hours prior to the transference of the egg. It must therefore be supposed that the depression determines the course of the maturation of the egg prior to that time.

VII. SUMMARY.

1. The two series of observations on Cladocera showing (a) that a state of depression produced by unfavourable external conditions in *Daphnia* females kept in cultures causes a change from parthenogenetic to gamogenetic propagation, and (b) that gamogenesis in nature is accompanied by a state of depression in the populations in question, go to prove the correctness of the hypothesis that in nature the transition from parthenogenesis to gamogenesis is caused by the influence of unfavourable external conditions. Unfavourable external conditions cause states of depression in the females and thereby the change in the mode of reproduction and so also a change in sex-determination. (Section II.)

2. A number of earlier and more recent laboratory experiments have given results which support this depression hypothesis. (Section III.)

3. Investigations in nature on parthenogenesis and gamogenesis in the Cladocera

and simultaneous observations on other functions in these animals likewise afford support for the depression hypothesis. (Section IV.)

4. The reproductive cycle of the Rotifera is compared with that of the Cladocera. There is a good deal of evidence which would seem to indicate that in the Rotifera transition from parthenogenesis to gamogenesis—and so also the sex determination associated with it—is likewise caused by a state of depression, and that this state of depression may be induced by the influence of various external factors. (Section V.)

5. A general survey is taken of some of the biological peculiarities characterising the state of depression associated with the change from parthenogenesis to gamogenesis. (Section VI.)

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Fig. 12. Printed by permission of the publishers of *Zoologica*, Heft 67, p. 350.

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THE HAEMOCYANINS

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As yet we know comparatively little about haemocyanin, especially with regard to its relation to oxygen. It is a matter which would well repay investigation to determine whether it has the remarkable properties which haemoglobin has in this respect, properties which are at present unique.

(Bayliss, *Principles of General Physiology*, 1915.)

CONTENTS.

	PAGE
I. Introduction	176
II. The chemical properties of haemocyanin	176
(1) Chemical composition	176
(2) The prosthetic group	178
(3) The molecular weight	180
(4) Molecular dimensions and symmetry	182
(5) Partial specific volume	182
(6) The isoelectric point	182
(7) Solubility	182
(8) Crystallisation	184
(9) The combination of haemocyanin with acid and base	185
(10) Viscosity	189
III. Optical properties	189
(1) Index of refraction	189
(2) The absorption of light by haemocyanin	190
(3) The scattering of light	190
(4) The spectrum of the chromatic group	192
(5) Absorption in the ultra-violet	193
(6) Beer's law	194
IV. The properties of the prosthetic group	194
(1) Oxygenation	195
(2) The equilibrium of haemocyanin with oxygen	195
(3) The kinetics of oxygenation	202
(4) Methaemocyanin	202
(5) The reaction of the prosthetic group with substances other than oxygen	203
V. Specificity and nomenclature	205
VI. Physiological considerations	205
VII. Summary	208
References	209

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I. INTRODUCTION.

THE haemocyanins are proteins occurring in solution in the blood of certain invertebrates. Together with the haemoglobins, chlorocruorins, and haemerythrin, the haemocyanins comprise a group of substances unique in uniting with oxygen in a way so labile that they serve in the transport of oxygen by the blood. Unlike the other members of this group, the haemocyanins are characterised by the presence of copper as part of their composition. The property of combining reversibly with oxygen apparently depends upon a prosthetic group of which the copper forms an essential part.

The earlier literature relating to the haemocyanins was reviewed by Quagliariello (1923, 1924) and Dhéré (1928). Subsequently a considerable volume of precise work has appeared which not only corrects or extends many of the earlier observations, but contributes to a number of questions scarcely raised by the earlier workers. At the present time our knowledge of the haemocyanins compares favourably in many respects with that of the haemoglobins. The purpose of the present review is to collect these recent data which define the properties of this group of substances.

Haemocyanins occur in the blood of molluscs and arthropods. Detailed studies have been made on haemocyanins isolated from the cephalopods (*Octopus*, *Sepia*, *Loligo*), the gastropods (*Helix*, *Busycon*), the decapod Crustacea (*Cancer*, *Homarus*, *Maia*, *Palinurus*, etc.) and the Xiphosura (*Limulus*). The blood of certain spiders appears to contain haemocyanin (Wilson, 1901), and Svedberg and Hedenius (1933) have confirmed Lankester's (1884) observation that this substance occurs in the blood of scorpions. Cuénot (1891) lists a number of gastropods and lamellibranchs of which the blood is considered to contain haemocyanin, but the identity in these cases is not established by isolation or chemical analysis, but rests merely on the blue colour of the body fluids. Svedberg and Hedenius have examined the blood of a great number of lamellibranchs by means of the ultra-centrifuge without finding haemocyanin.

It has been suggested by Muttkowski (1921) on the basis of the qualitative demonstration of copper in the blood of certain insects, that these contain haemocyanin. We have measured the concentration of copper in the blood of the Florida grasshopper and have found quantities so small that if it is present as haemocyanin the latter can have no importance in the transport of oxygen by the blood.

In no case is haemocyanin known to occur in blood corpuscles or within tissue cells.

II. THE CHEMICAL PROPERTIES OF HAEMOCYANIN.

(1) *Chemical composition.*

The best data on the elementary composition of the haemocyanins are given in Table I. They are based on the analyses of Hernler and Philippi (1930, 1933), made upon materials purified by the best available methods. The copper contents shown by these analyses agree closely with the earlier measurements made upon

Table I. *Elementary composition of various haemocyanins.*

	C %	H %	N %	S %	Cu %
Gastropods					
<i>Helix pomatia</i>	53.4	6.9	15.15	0.76	0.24
<i>Busycon canaliculatum</i>	53.5	6.65	15.9	1.23	0.245
Cephalopods					
<i>Octopus vulgaris</i>	53.4	6.95	15.9	1.04	0.25
<i>Loligo pealei</i>	52.75	6.8	15.75	1.19	0.26
Xiphosura					
<i>Limulus polyphemus</i>	53.4	6.9	16.9	1.10	0.173
Crustacea					
<i>Homarus americanus</i>	53.07	6.85	16.78	0.90	0.187

the haemocyanin of *Helix* by Dhéré (1919*b*), Burdel (1922), and Begemann (1924); of *Limulus* by Redfield, Coolidge and Shotts (1928); of *Busycon* and *Loligo* by Montgomery (1930). Earlier analyses of the copper content of the haemocyanin of *Limulus* by Alsberg and Clark (1910), of *Octopus* by Henze (1901), and of *Sepia*, *Cancer* and *Homarus* by Griffiths (1892) cannot be reconciled with these measurements; the determinations of the other elements made by these authors do not deviate widely from those of Hernler and Philippi.

The general proportions of C, H, O, N and S in the haemocyanins are a reflection of their protein nature. It is noteworthy that the haemocyanins of the arthropods, *Limulus* and *Homarus*, contain definitely more nitrogen than do those of the molluscs. A similar distinction occurs in the case of the copper content—the arthropod haemocyanins containing about 0.18 per cent., whereas the molluscan haemocyanins yield approximately 0.25 per cent. copper. Were it not for the distinct differences in the physical properties of the haemocyanins within each of these groups, one might conclude that within each phylum the haemocyanins were similar, if not identical.

The haemocyanin of *Busycon canaliculatum*, which was described by Mendel and Bradley (1907) under the name *haemosycotypin*, was thought by these authors to contain zinc as well as copper. Recent spectrographic analysis of this, as well as other species of haemocyanin by Gatterer and Philippi (1933), has failed to confirm the presence of zinc or of any other heavy metal except copper in purified preparations of the proteins. Unpublished experiments made by Dr Beecher in the author's laboratory were also unsuccessful in demonstrating the presence of zinc in purified preparations of *Busycon* haemocyanin, although this element could be detected in preparations of whole blood.

Further knowledge of the composition of the haemocyanins is fragmentary. Henze (1904) estimated the distribution of nitrogen in the haemocyanin of *Octopus vulgaris* to be as follows:

	%
NH ₃ nitrogen ...	5.78
Humin nitrogen ...	2.67
Diamid nitrogen ...	27.65
Monoamid nitrogen ...	63.39

Van Slyke (1911-12) obtained the following results from a study of the distribution of nitrogen in *Limulus* haemocyanin:

	%
NH ₂ nitrogen	5.95
Melanin nitrogen	1.65
Cystine nitrogen	0.80
Arginine nitrogen	15.73
Histidine nitrogen	13.23
Lysine nitrogen	8.49
Amino nitrogen in filtrate	51.30
Non-amino nitrogen in filtrate (prolin, oxyprolin, $\frac{1}{2}$ tryptophane)	3.80

Sulphur occurs in all haemocyanins in quantities which appear to vary from species to species. Prof. H. T. Clarke (personal communication) has found that less than half of the sulphur in *Limulus* haemocyanin may be accounted for as cystine sulphur.

(2) *The prosthetic group.*

Particular interest attaches to the chemical nature of the portion of the haemocyanin molecule which contains the copper and which presumably functions as an oxygen carrier. The phenomenal success made by students of this problem in the case of haemoglobin has stimulated an analogous investigation of the prosthetic group of haemocyanin.

Henze (1901) observed that treatment of *Octopus* haemocyanin with acid led to a separation of the copper from the protein. He concluded that the copper was not present in a stable organic binding, as is the iron in haemoglobin, but is present as a copper albuminate. Henze's view was supported by Kobert (1903) and Alsberg and Clark (1910). Roche (1930) has also concluded from a comparison of the physical properties of the protein before and after removal of the copper by treatment with acid that one cannot accept the presence in haemocyanin of a prosthetic group analogous to that of haemoglobin, but more recently he appears to have abandoned this view (Roche and Dubouloz, 1933).

On the other hand, by adopting the methods used in separating the phyllins of chlorophyll, Philippi (1919) secured from *Helix* haemocyanin treated with strong alkali a dark green precipitate which contained 7 per cent. copper. This product gave an intense pyrrol reaction with the pine splint test. The result was of great interest, as pyrrols form the basis of the structure of the prosthetic group of haemoglobin. Dhéré and Schneider (1922*a*) also prepared this product and proposed to call it *hématoctyanine*. Dhéré and Baumeler (1928, 1929) were unable to detect in this product any derivatives giving the red fluorescence characteristic of porphyrins, as would be expected were the prosthetic group of haemocyanin similar to that of haemoglobin.

Schmitz (1930, 1931*a, b*) secured a product by decomposing *Octopus* haemocyanin with alkali which he designated *Hämocuprin*. This substance has the composition C 45.04, H 6.99, N 12.58, Cu 6.27 per cent. He believes it to be a tetrabasic organic acid containing copper in complex binding, and finds no evidence of porphyrin structure.

Conant and Humphrey (1930) likewise secured an insoluble copper compound by treating *Limulus* haemocyanin according to Philippi's procedure. After further purification a black amorphous powder containing practically all the copper of the original haemocyanin was obtained. This product had the following composition:

			%
Cu	21.5
N	9.2
C	39.5
H	5.6
S	8.0

The available evidence indicated that this product is a complex salt of an amino acid which contains sulphur.

By the action of ammonia on this pigment an amorphous, colourless solid was obtained which was free from copper and sulphur and gave no evidence of containing pyrrol nuclei.

Conant, Dersch, and Mydans (personal communication) have found that this material is a polypeptide composed of leucine, tyrosine, and at least one other amino acid tentatively identified as serine. Basic amino acids, tryptophane, and histidine are absent. The sulphur in the original black powder is present neither as cystein, cystin, nor methionine. Since the sulphur compound and the copper are removed when the black material is treated with ammonia, it is probable that the sulphur compound is linked through the copper and not by a peptide linkage to the polypeptide. They suggest that the prosthetic group of *Limulus* haemocyanin is a complex copper salt of an unknown sulphur compound and a polypeptide, consisting of one molecule each of leucine and tyrosine and three of serine.

Conant and his collaborators have evidence that this complex is in reality a prosthetic group of haemocyanin, and is not formed by the combination of fragments into which the original protein is decomposed by the action of alkali. They are unable to obtain a product similar to Schmitz' *Hämocuprin* by applying his procedures to *Limulus* haemocyanin, but secure a product similar to *Hämocuprin* by prolonged treatment of their black powder with normal sodium hydroxide. It appears, therefore, that there may be some differences between *Octopus* haemocyanin and that of *Limulus* which alter the ease of decomposition of the complex copper compound by alkali, but that in both cases essentially the same sort of prosthetic group is present. Differences in the ease with which copper may be separated from different haemocyanins is also indicated by the fact that the haemocyanins of *Octopus*, *Limulus*, and *Busycon* give the biuret reaction on the addition of alkali, but that of *Helix* does not give a positive reaction unless a trace of copper is also added (Dhéré, Baumeler and Schneider, 1929).

Finally, Laporta (1932) has secured a product which he considers identical with Schmitz' *Hämocuprin* by treating *Octopus* haemocyanin with acetone and acid. This product contains copper and gives the pine splint test of pyrrol. The product obtained by this method from the haemocyanins of the octopus, crab, and snail have the same ultra-violet absorption spectrum (Roche and Dubouloz, 1933).

This is all that can be said concerning the chemical composition of the haemocyanins and the structure of the prosthetic group. From the standpoint of the organic chemist much remains to be done.

(3) *The molecular weight.*

The minimal molecular weight of the haemocyanins, estimated as the smallest weight which would contain one atom of copper, is about 25,000 in the case of the molluscan haemocyanins and 37,000 in the case of those of the arthropods. These are the largest known minimal molecular weights to be based on purely analytical data. As will be pointed out below, the quantity of haemocyanin which unites with one molecule of oxygen contains two atoms of copper. On the basis of their oxygen-binding power the minimal molecular weights of the two classes of haemocyanin become 50,000 and 74,000.

Experiments by Cohn (1925) on the filtration of proteins through collodion membranes placed *Limulus* haemocyanin as intermediate in size between haemoglobin and pseudoglobulin, which would indicate that its true molecular weight is equal to the minimal weight 74,000 or a value twice as great. The equilibrium between purified *Limulus* haemocyanin and oxygen may be described by the mass law, assuming that each molecule of haemocyanin unites with a single molecule of oxygen. This would be the case were the molecular weight 74,000.

While there is thus some evidence that the molecule of *Limulus* haemocyanin contains but two atoms of copper and has a molecular weight comparable in size to that of haemoglobin and many other proteins, other evidence suggests that the haemocyanin molecules are much larger, or at least that the units of minimal molecular weight are associated in aggregations of uniform size but of much greater bulk. Dhéré and Baumeler (1926) observed that haemoglobin would pass through collodion membranes which retained *Helix* haemocyanin and concluded that the haemocyanin molecules are larger than those of haemoglobin (molecular weight 66,800). The equilibrium of haemocyanin with oxygen, in the case of the native blood of several species, can be accounted for under certain circumstances on the assumption that the haemocyanin forms a stable oxygenation product only when it unites with four molecules of oxygen. This assumption necessitates a haemocyanin molecule containing eight atoms of copper and consequently possessing a molecular weight of 200,000 in the case of the molluscs, and 300,000 in the case of the arthropods. The equilibrium between haemocyanin and cyanide (see p. 203) and oxidation-reduction equilibria (Conant, Chow and Schoenbach, 1933) also suggest a haemocyanin unit of about this size.

The evidence of Svedberg and his collaborators, obtained by means of studies of the sedimentation velocity and equilibrium of haemocyanin solutions under centrifugal force, show that the haemocyanins may exist as particles of uniform size having the weights shown at the top of p. 181.

These are values unapproached by other proteins save for the respiratory proteins of the blood of certain worms, in which haemoglobin and chlorocruorin occur in solution (Svedberg and Eriksson, 1932*a*).

<i>Helix</i> haemocyanin	...	5,000,000 (Svedberg and Chirnoaga, 1928)
<i>Octopus</i> haemocyanin	...	2,000,000 (Svedberg and Eriksson, 1932 <i>b</i>)
<i>Limulus</i> haemocyanin	...	1,300,000 ¹
<i>Homarus</i> haemocyanin	...	640,000 (Svedberg and Eriksson, unpublished)
<i>Palinurus</i>	360,000 (Svedberg and Eriksson, unpublished)

Svedberg has published measurements of the sedimentation constant of the haemocyanins of a large number of species. The sedimentation constant is defined as the velocity of sedimentation in a centrifugal field of unit strength, reduced to water of 20° as solvent. This constant is a function of the mass and shape of the molecule and is thus characteristic of the molecular species studied. The sedimentation constants of the haemocyanins examined are given in Table II. These

Table II. *Sedimentation constants of various haemocyanins.*

Species	Sedimentation constant $S_{20^\circ} \times 10^{13}$	Species	Sedimentation constant $S_{20^\circ} \times 10^{13}$
Crustacea		Gastropoda (cont.)	
<i>Palaemon fabrici</i>	16.9	<i>Littorina littorea</i>	99.8
<i>Pandalus borealis</i>	16.9	<i>Buccinum undatum</i>	99.8
<i>Palinurus vulgaris</i>	16.9	<i>Neptunea antiqua</i>	99.8
<i>Eupagurus bernhardus</i>	16.9	<i>Limnea stagnalis</i>	99.8
<i>Pagurus striatus</i>	16.9	<i>Achatina fulva</i>	99.8
<i>Nephrops norvegicus</i>	23.4	<i>Helix pomatia</i>	99.8
<i>Homarus vulgaris</i>	23.4	<i>H. arbustorum</i>	99.8
<i>Astacus fluviatilis</i>	23.4	<i>H. nemoralis</i>	99.8
<i>Hyas araneus</i>	23.4	<i>H. hortensis</i>	99.8
<i>Maia squinado</i>	23.4	<i>Limax maximus</i>	99.8
<i>Cancer pagurus</i>	23.4	<i>L. agrestis</i>	99.8
<i>Carcinus maenas</i>	23.4	<i>Arion ater</i>	60.8
<i>Squilla mantis</i>	23.4		
<i>Calocharis macandrae</i>	34.1	Cephalopoda	
Arachnomorpha		Decapoda	
<i>Limulus polyphemus</i>	35.7	<i>Loligo vulgaris</i>	57.1
<i>Euscorpius</i> sp.	34.1	<i>Sepiola oweniana</i>	57.1
Amphineura		<i>Rossia macrosoma</i>	57.1
<i>Tonicella marmorea</i>	60.8	<i>Sepia officinalis</i>	57.1
Gastropoda		Octopoda	
<i>Paludina vivipara</i>	99.8	<i>Octopus vulgaris</i>	51.1
<i>P. contecta</i>	99.8	<i>Eledone cirrosa</i>	51.1
		<i>E. moschata</i>	51.1

haemocyanins are all characterised by high sedimentation constants and have therefore high molecular weights. Within each well-defined group all the species have, as a rule, practically the same sedimentation constant.

These enormous molecules, which each comprise from 20 to 80 oxygen equivalents, appear to be made up of associations of smaller units. *Helix* haemocyanin, at reactions removed from the isoelectric point, gives sedimentation velocity measurements which indicate that under these conditions the particles disintegrate into smaller particles of undetermined weight (Svedberg and Heyroth, 1929*b*). *Limulus* haemocyanin appears to disintegrate into smaller units on dilution to

¹ Prof. Svedberg informs me that recent measurements indicate that the molecular weight of *Limulus* haemocyanin is 1,300,000 rather than 2,000,000 as reported by Svedberg and Heyroth (1929*a*).

concentrations of 0.1 per cent. or less (Svedberg and Heyroth, 1929*b*). The haemocyanin of *Homarus* dissociates at high alkalinities into molecules of half the size of those which exist at neutral reactions. In *Octopus* haemocyanin there is present, both in the case of the blood and of purified preparations, two components, one of uniform molecular weight of 2,000,000, the other of smaller size. The size of the latter and the ratio of the two components both vary widely and reversibly with hydrogen-ion concentration.

(4) *Molecular dimensions and symmetry.*

From considerations involving diffusion constants, molecular weight, and viscosity Svedberg and his collaborators conclude that the molecule of *Helix* haemocyanin is spherical and has a radius of 12×10^{-7} cm. The dissymmetry number of *Helix* haemocyanin is 1.05. Prof. Svedberg informs me that recent work indicates that the molecules of *Limulus* and *Octopus* haemocyanin are also nearly spherical and that the published dissymmetry numbers for these proteins are subject to revision.

(5) *Partial specific volume.*

Svedberg and his collaborators have found that the partial specific volumes of various haemocyanins are almost identical and are independent of concentration. The values obtained are:

<i>Helix pomatia</i>	0.738 (Svedberg and Chirnoaga, 1928)
<i>Limulus polyphemus</i>	0.735 (Svedberg and Heyroth, 1929 <i>a</i>)
<i>Octopus vulgaris</i>	0.740 (Svedberg and Eriksson, 1932 <i>b</i>)

The partial specific volume of most other proteins is slightly greater than these values (Svedberg, 1930*a*).

(6) *The isoelectric point.*

The isoelectric points of the various haemocyanins obtained by the method of cataphoresis are recorded in Table III. The differences in these values indicate that the haemocyanins of the different species are distinct chemical substances. Even members of the same genus—as in *Paludina* and *Helix*—have different isoelectric points. For the genus *Helix*, containing several sub-genera, the isoelectric points lie closer together within the sub-genus.

(7) *Solubility.*

The solubility of the haemocyanins of *Limulus*, *Octopus*, and *Helix* in distilled water, after the addition of various quantities of HCl or NaOH, has been measured by Roche (1930, 1932). His results are shown in Fig. 1. Aside from this, no adequate studies have been made of the solubility of haemocyanins. Nevertheless, there exists considerable qualitative information which seems to characterise the haemocyanins of different species.

The haemocyanins all appear to be globulins (Quagliariello, 1924). They are soluble in dilute salt solutions but are insoluble at certain hydrogen-ion con-

Table III. Isoelectric point, pH value at which solubility is minimal and slope of the mobility-pH curve ($\frac{du}{dpH}$) of various haemocyanins.

Species	Isoelectric point	pH of minimal solubility	$\frac{du}{dpH} \times 10^{-5}$	Authority
<i>Octopus vulgaris</i>	4.7	—	—	Quagliariello (1920b)
<i>O. vulgaris</i>	4.8	4.8-5.0	—	Roche (1930)
<i>Eledone cirrosa</i>	4.6	—	—	Pedersen (1933)
<i>Helix pomatia</i>	5.05	—	8.1	Svedberg (1930a); Pedersen (1933)
<i>H. pomatia</i>	5.3	—	—	Stedman and Stedman (1927)
<i>H. pomatia</i>	—	5.2	—	Roche (1932)
<i>H. nemoralis</i>	4.63	—	11.4	Pedersen (1933)
<i>H. hortensis</i>	4.57	—	12.1	Pedersen (1933)
<i>H. arbustorum</i>	5.50	—	7.6	Svedberg (personal communication)
<i>Paludina vivipara</i>	4.71	—	10.8	Svedberg (personal communication)
<i>P. coniecta</i>	4.63	—	11.4	Svedberg (personal communication)
<i>Littorina littorea</i>	4.34	4.4	12.8	Svedberg (personal communication)
<i>Buccinum undatum</i>	4.61	4.6	13.7	Svedberg (personal communication)
<i>Achatina fulva</i>	5.03	—	7.8	Svedberg (personal communication)
<i>Cancer</i> sp.	4.7	—	—	Stedman and Stedman (1927)
<i>C. pagurus</i>	4.65	—	16.0	Pedersen (1933); Svedberg (personal communication)
<i>Homarus</i> sp.	—	4.8	—	Stedman and Stedman (1926a)
<i>H. vulgaris</i>	4.95	—	18.0	Pedersen (1933); Svedberg (personal communication)
<i>Astacus fluviatilis</i>	4.93	4.8	12.1	Pedersen (1933)
<i>Limulus polyphemus</i>	6.4	—	—	Svedberg (1930a)
<i>L. polyphemus</i>	—	6.2-6.5	—	Roche (1930)
<i>L. polyphemus</i>	—	6.3	—	Stedman and Stedman (1926a)

centrations if the concentration of electrolyte is sufficiently low. The data recorded in Table III show that the hydrogen-ion concentration at which solubility is minimal coincides with the isoelectric point, as measured by the method of cataphoresis.

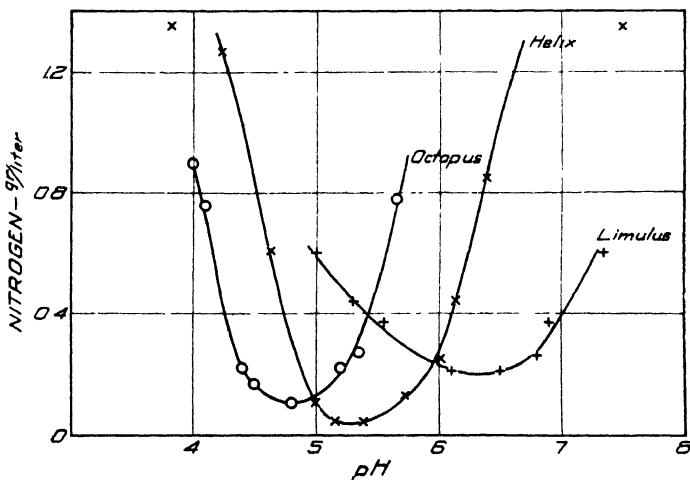


Fig. 1. Solubility of the haemocyanins of *Limulus*, *Octopus*, and *Helix* in distilled water, after the addition of various quantities of HCl or NaOH.

The various haemocyanins show great quantitative variation in the effect of salts on their solubility. *Busycon* haemocyanin may be precipitated at the isoelectric point in the presence of considerable salt, and one may isolate the protein from the blood merely by diluting the latter with a few volumes of water and then adding sufficient dilute acid. *Limulus* haemocyanin must be dialysed until almost free of salt before it can be so separated. *Helix* haemocyanin is even more soluble than *Limulus* in dilute salt solutions (Svedberg and Heyroth, 1929*a*). Pedersen (1933) observed that the haemocyanins of *Paludina vivipara*, *P. contecta*, *Achatina fulva*, *Helix pomatia*, *H. nemoralis*, and *H. hortensis* are soluble in 0.02 *M* sodium acetate buffers at the isoelectric point, whereas the haemocyanins of *Buccinum undatum*, *Littorina littorea*, and *Astacus fluviatilis* were insoluble.

Limulus haemocyanin is insoluble in the presence of salt at acid reactions (Redfield and Mason, 1928). The haemocyanins of different species appear to differ in the concentration of salt required to "salt out" the protein. *Limulus* haemocyanin is precipitated in half-saturated ammonium sulphate, whereas full saturation is required for the salting out of *Octopus* haemocyanin (Alsberg and Clark, 1910). The results of such experiments depend upon the hydrogen-ion concentration and temperature and here again extensive measurements under controlled conditions are lacking.

Crafaleanu (1918) observed that *Octopus* haemocyanin which has been kept in the crystalline condition in half-saturated ammonium sulphate for a time becomes insoluble in distilled water and proposed the name *parahaemocyanin* for this modification. The haemocyanin of the squid displays the same phenomenon (Montgomery, 1930).

(8) Crystallisation.

The conditions under which haemocyanin may be obtained in crystalline form are reviewed by Dhéré (1919*b*). The haemocyanin of the octopus was first crystallised by Henze (1901), employing the method of Hofmeister and the improvement introduced by Hopkins and Pinkus. On the addition of acetic acid to a saturated solution of haemocyanin dissolved in strong ammonium sulphate, the protein becomes less soluble and crystallises out. The haemocyanin of *Loligo* may be crystallised in a similar fashion. Montgomery (1930) has found that raising the temperature decreases the solubility of the haemocyanin and causes crystallisation without necessitating the addition of acid. Maximum yields were obtained by combining the two procedures.

The haemocyanin of *Helix*, which is insoluble in the absence of electrolytes, has been crystallised by Dhéré by prolonged dialysis. In another procedure an electric current is passed through a dialysed solution, causing crystallisation of the haemocyanin to occur at the positive pole. If one adds an excess of these crystals to a very dilute (*N*/500) solution of sodium sulphate a saturated solution is obtained from which an abundant crystallisation takes place on standing. Dhéré and Baumeler (1929) discovered that if papain is added to filtered *Helix* blood, or

alkaline solutions of crystalline haemocyanin, and the solutions allowed to stand, after twenty-four hours numerous needle-shaped crystals appear. These will dissolve, but with difficulty, in water, and the solutions are similar in many ways to those of the original haemocyanin. Philippi and Hernler (1930) determined the elementary composition of such crystals and concluded that they were probably pure haemocyanin, but without definitely excluding the possibility that they might be slightly modified addition or decomposition products of the original protein.

The haemocyanin of *Palinurus* may be obtained in crystalline form by a similar method. If the serum is placed in an electric field an amorphous precipitate forms at the anode. The portion of the liquid containing the precipitate is diluted with sodium chloride to a final concentration of $N/5$ which causes the precipitate to dissolve. On standing, crystals of haemocyanin are formed.

The haemocyanins of *Limulus* and *Busycon* have not been obtained in crystalline form (Dhéré, Baumeler and Schneider, 1929).

The form of the crystals is very variable, being cubic(?), quadratic, and hexagonal (or rhombic?) in the snail, cubic and rhombohedral in *Palinurus*. Those of the cephalopod haemocyanins are long needles. The form of the crystals varies greatly with the method of preparation.

The crystals of *Octopus* haemocyanin (Henze, 1901) and those of the squid are doubly refractive.

(9) *The combination of haemocyanin with acid and base.*

The amphoteric character of the haemocyanins arises from their protein nature. It is important not only as a means of chemical characterisation, but because the ability to combine with base enables the haemocyanins to perform a vital physiological function in the transport of carbon dioxide in respiration, and in buffering the blood.

Quagliariello (1916) showed that relatively large amounts of acid or alkali must be added to the blood of certain invertebrates, containing haemocyanin, in order to alter its reaction significantly. The equilibrium of the blood of a number of invertebrates with carbon dioxide was studied by Parsons and Parsons (1923), who pointed out that those bloods which combine with carbon dioxide in quantity are rich in haemocyanin. These results were confirmed in the case of *Limulus* blood by Redfield, Coolidge and Hurd (1926) and Redfield, Humphreys and Ingalls (1929). The latter conclude that substances other than haemocyanin account for only a small part of the buffering of *Limulus* blood.

Nitzescu and Cosma (1927), on the other hand, measured the carbon dioxide dissociation curve of the blood of *Helix pomatia*. They observed that although the amount of carbon dioxide bound was high, the blood was a weaker buffer than rabbit serum, which contains an equivalent amount of protein. They concluded that proteins do not play the principal rôle in the acid-base equilibrium of *Helix* blood.

The haemocyanin of *Limulus* binds about 160×10^{-5} mol of acid per gram.

The total base-binding capacity has not been precisely determined, but is of a similar magnitude. Redfield and Mason (1928) pointed out that the acid-binding power agrees closely with that calculated from the dibasic amino acids present in its composition. Roche (1932) has shown that the haemocyanin of *Helix pomatia* binds 91×10^{-5} mol of HCl and 61×10^{-5} mol of NaOH per gram, quantities notably inferior to those characterising *Limulus* haemocyanin. The lower diamino-acid content suggested by this result is possibly correlated with the lower nitrogen content of *Helix* haemocyanin as compared with that of *Limulus*.

From the oxygen-binding power of the haemocyanins it may be estimated that a quantity of the protein which combines with one molecule of oxygen contains in the case of *Limulus* 117 groups capable of neutralising HCl. In the case of *Helix* the equivalent acid-combining capacity is 45, the equivalent base-combining capacity 30.

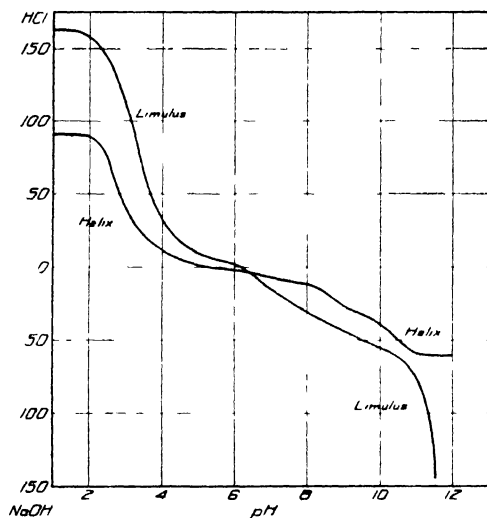


Fig. 2. Titration curves of the haemocyanins of *Helix* and *Limulus*. Ordinates, mols of HCl or NaOH added per 10^5 grams protein.

Complete titration curves of the haemocyanins of *Limulus* and *Helix*, based on the measurements of Redfield and Mason (1928), Redfield, Humphreys and Ingalls (1929), and Roche (1932), are shown in Fig. 2. These curves show certain individual differences as do the less complete titration curves for the haemocyanins of *Homarus vulgaris* (Stedman and Stedman, 1926a), of *Busycon canaliculatum* (Redfield and Ingalls, 1932), and the octopus (Roche, 1930). They are, however, similar in form to the titration curves of other proteins such as those published by Cohn (1925).

Limulus haemocyanin reacts with acid as though it were a univalent base with a pK value of 3.3 (Redfield and Mason, 1928); for *Helix* the pK value in the acid range is 3.2 (Roche, 1932). The titration curves in the alkaline range are more

complicated because, apparently, the various base-binding groups differ in the values of their dissociation constants. Roche considers that three dissociation constants may be distinguished in *Helix* haemocyanin characterised by pK values of 10.5, 8.6 and 7.6. The latter value changes to 7.0 when the haemocyanin becomes oxygenated. In *Limulus* haemocyanin the greater part of the alkali is bound at reactions in the neighbourhood of pH 11. Considerable buffer action is observed at more neutral reactions corresponding to the lower pK values distinguished by Roche.

The low base-binding capacity of *Helix* haemocyanin in the physiological range, as compared to that of *Limulus*, accounts for the poor buffering observed by Nitzescu and Cosma (1927) in the blood of the snail.

The individuality of the various haemocyanins in regard to their acid-base-binding power is brought out by the way in which the mobility of the protein in an electric field varies with pH in the neighbourhood of the isoelectric point. The values of the slope of the mobility- pH curve $\left(\frac{du}{d pH} \times 10^{-5}\right)$ given in Table III are based on the measurements of Pedersen (1933) and on unpublished values kindly given by Prof. Svedberg.

The titration curve of *Limulus* haemocyanin does not coincide with that of the serum of this animal. The addition of sodium chloride to the haemocyanin alters the titration curve of *Limulus* and *Busycon* haemocyanin in such a way as to indicate that the pK values of the base-binding groups are decreased about 0.5 pH unit (for the addition of 0.5 M NaCl). Similar effects have been observed in the case of other proteins (Sørensen, Linderstrøm-Lang and Lund, 1927; Simms, 1929). By dissolving the haemocyanin in a mixture of salts approximating to that found in the serum a titration curve was obtained closely resembling that of the serum. It was on the basis of this observation that it was concluded that the haemocyanin was the principal buffer of the *Limulus* blood.

The buffer value of reduced *Limulus* haemocyanin is nearly constant over the pH range from 6.0 to 10 and amounts to 0.15 mol per gram of protein. This value is nearly the same as that of reduced haemoglobin which varies between 0.143 and 0.169 in different species (Redfield, 1933). The addition of salt increases the buffer value of *Limulus* haemocyanin to about 0.23 mol per gram in the physiological range of hydrogen-ion activity.

The pK values of some base-binding groups of certain haemocyanins are also altered by oxygenation. This phenomenon is analogous to that observed in many species of haemoglobin. Kerridge (1926) found in studying the carbon dioxide dissociation curves of the blood of *Maia squinado* that the oxygenated haemocyanin was a stronger acid than the reduced haemocyanin. As a result, in the range of pH 6.3–6.9 the reduced blood combined approximately 0.5 millimol more carbon dioxide per litre than did the oxygenated blood at the same pH value. She estimated that in this range the quantity of carbonic acid displaced on oxygenation was equivalent to the quantity of oxygen bound. Qualitatively similar results have been obtained by Parsons and Parsons (1923) with the blood of

Octopus macropus and by Redfield, Coolidge and Hurd (1926) with that of *Loligo pealei*.

In discussing the analogous phenomenon displayed by haemoglobin, Henderson (1920) pointed out that it followed as a physical necessity that since carbon dioxide (or other acids) diminishes the affinity of haemoglobin for oxygen, the combination of oxygen with haemoglobin must reduce the tendency of the blood to take up carbon dioxide. The foregoing observations on the haemocyanins of *Maia*, *Octopus*, and *Loligo* are in conformity with this consideration since it has been shown, in the case of *Maia* (Hogben, 1926) and *Loligo* (Redfield, Coolidge and Hurd, 1926), that the oxygen equilibrium is shifted by changes in acidity in a manner similar to that of haemoglobin.

The bloods of *Limulus*, *Busycon* (Redfield, Coolidge and Hurd, 1926) and *Helix* (Hogben, 1926) differ from those of the Crustacea and cephalopods and from haemoglobin in that at physiological reactions the affinity of the haemocyanin for oxygen is increased by the addition of acid. From the principle set forth by Henderson one would expect these bloods to combine with more carbon dioxide (or other acid) when oxygenated than when reduced. This result is obtained with *Busycon* serum. In the case of *Limulus* no difference could be detected in the carbon-dioxide dissociation curves of oxygenated and reduced blood. Studies by Mr Shack of the complete titration curves of the serum and of the purified haemocyanin of *Limulus* have failed to show any difference in the behaviour of oxygenated and reduced samples. It is possible that the effect expected on theoretical grounds is present but is too small to be detected.

The titration curves of the oxygenated and reduced haemocyanin of *Helix* have been examined by Roche (1932), who concludes that the base-binding groups having a pK value of 7.6 in the reduced haemocyanin possess a pK value of 7.0 when the protein is oxygenated. Oxygenation thus makes the haemocyanin appear to act like a stronger acid—as it does with haemoglobin. This result is not what one would expect from the data on the oxygen equilibrium at various reactions, for Stedman and Stedman (1928) concluded that purified *Helix* haemocyanin was uninfluenced in its affinity for oxygen by alterations in hydrogen-ion concentration. A further analysis of their data led Redfield (1930*b*) to suggest that increasing acidity favoured the union of *Helix* haemocyanin with oxygen—a result directly opposed to the expectation raised by Roche's observations on the titration curves.

By making the assumption that the difference in the titration curves of oxygenated and reduced haemocyanin is due to a shift in the pK value of a single acid-binding group for each molecule of oxygen combined by the haemocyanin, Mr Shack has calculated the change in the value of the pK of this group. With the exception of *Helix* haemocyanin, the anomalous behaviour of which is pointed out above, the magnitude of this change parallels the magnitude of the change in oxygen equilibrium brought about by altering the hydrogen-ion activity. This parallelism is illustrated in Table IV, in which the change in pK due to oxygenation is compared with the maximum change in oxygen pressure required to half-saturate the haemocyanin which may be produced by altering the acidity of the solutions.

Table IV. Comparison of the change in pK (ΔpK) of the acid-binding group of haemocyanin and haemoglobin influenced by oxygenation, and the change in oxygen pressure (Δp_{50}) required to half-saturate the protein due to altered acidity.

	ΔpK	Δp_{50} mm. Hg
Lobster haemocyanin		
Native serum (Shack, 1933)*	0.758	—
Dialysed serum (Stedman, 1926a)	—	370
Maia haemocyanin		
Native serum (Kerridge, 1926)	0.50	—
" " (Hogben, 1926)	—	71
Horse haemoglobin		
Dialysed	0.35	17
Limulus haemocyanin		
Purified haemocyanin (Shack)*	0	—
" " (Redfield, 1930b)	—	— 3
Native serum (Redfield and Ingalls, 1933)	—	— 12

* Unpublished data.

(10) Viscosity.

Observations on the viscosity of the haemocyanins of *Cancer* and *Homarus* have been made by Stedman and Stedman (1927). Their conclusion that a relation exists between the viscosity and the affinity of the protein for oxygen has been discussed by Redfield and Ingalls (1932). Svedberg and Heyroth (1929b) suggest that the change in viscosity of haemocyanin solutions may be due in part at least to the dispersion of the protein into smaller units such as they observe in *Helix* haemocyanin at reactions removed from the isoelectric point.

III. OPTICAL PROPERTIES.

(1) Index of refraction.

Quagliariello (1920a) has made a systematic investigation of the refractive index of solutions of *Octopus vulgaris* haemocyanin. He found that the refractive index of the solution is increased over that of the solvent 0.00197 for each gram present in 100 c.c. of solution. This value is uninfluenced by temperature (10–30° C.) or the presence of acids, bases, and salts. I have obtained practically identical values for the haemocyanins of *Busycon*, *Limulus*, and *Homarus* as the data in Table V show. These results are of little theoretical interest aside from showing the great similarity of the various haemocyanins in this regard, and demonstrating their general resemblance to other proteins. They form the basis, however, of a very convenient method of estimating approximately the concentration of haemocyanin solutions in the course of experimental work.

Table V. *Refractive index of haemocyanin solutions.*

Species	Concentration of haemocyanin %	Temp. ° C.	Solvent	Refractive index of solution	Refractive index of solvent	$\frac{N-N_0}{c}$
<i>Octopus vulgaris</i> *	1.134	—	0.05 NaOH	1.336166	1.333931	0.00197
	1.134	—	0.05 HCl	1.335938	1.333697	0.00198
	1.134	—	1 N NaCl	1.337088	1.334857	0.00197
<i>Burycon canaliculatum</i>	2.18	20	H ₂ O	1.33724	1.33300	0.00194
	10.97	24	H ₂ O	1.3543	1.3326	0.00198
	3.66	23	H ₂ O	1.34000	1.33279	0.00197
	1.083	23	H ₂ O	1.33494	1.33297	0.00198
<i>Limulus polyphemus</i>	4.90	15.2	H ₂ O	1.34303	1.33329	0.00198
	4.90	15.2	5 % KCl	1.34654	1.33682	0.00198
	7.26	23	H ₂ O	1.34715	1.33275	0.00198
<i>Homarus americanus</i>	11.85	20	H ₂ O	1.35668	1.33300	0.00200

* Selected from Quagliariello (1920a).

(2) *The absorption of light by haemocyanin.*

The earlier work on the absorption of light by haemocyanin was reviewed by Dhéré and Burdel (1919), and Dhéré, Baumeler and Schneider (1929) in papers in which they described original observations on the oxyhaemocyanin of *Helix*, *Sepia*, *Palinurus*, *Homarus*, and *Limulus* made with a spectrographic technique. Dhéré and Burdel pointed out that the apparent absorption of light by haemocyanin solutions is of a complex nature, and is due in part to colloidal phenomena, "réflexion lumineuse sur les grosses particules colloïdales," and in part to selective absorption by the molecule of oxyhaemocyanin. They found a band of maximal absorption in the visible range, the axis of which varied between 571 and 581 *mμ*. They concluded that variation in the situation of this axis depends not only on the electrolytes present in the solvent but also on the species of animal which yielded the pigment.

The employment of spectrophotometric technique by Vlès (1913), Quagliariello (1922), Begemann (1924), Svedberg and Chirnoaga (1928), Svedberg and Heyroth (1929a), and Redfield (1930a) enables the absorption of light by haemocyanin solutions to be described more precisely.

(3) *The scattering of light.*

Redfield showed that the apparent absorption of light in the visible part of the spectrum by specimens of blood containing reduced haemocyanin was due solely to the Tyndall effect, that is, the scattering of light by the constituents of the solution. This was demonstrated by the fact that the extinction coefficients of the reduced blood at various wave-lengths vary with the reciprocal of the fourth power of the wave-length. This relation was deduced by Lord Rayleigh for the loss of intensity through scattering when light is passed through a medium con-

taining particles small when compared to the wave-length. The data in Fig. 3 demonstrate this relationship. The light emerging from the solution at an angle of 90° from the incident beam, when viewed through a Nicol prism, is nearly completely polarised, as required by the theory of scattering. This fact indicates that haemocyanin solutions are not fluorescent, as claimed by Lankester (1871) and Jolyet and Regnard (1877).

The amount of light scattered by the haemocyanin present in blood varies greatly in different species, being largest in the gastropod *Busycon*, least in the

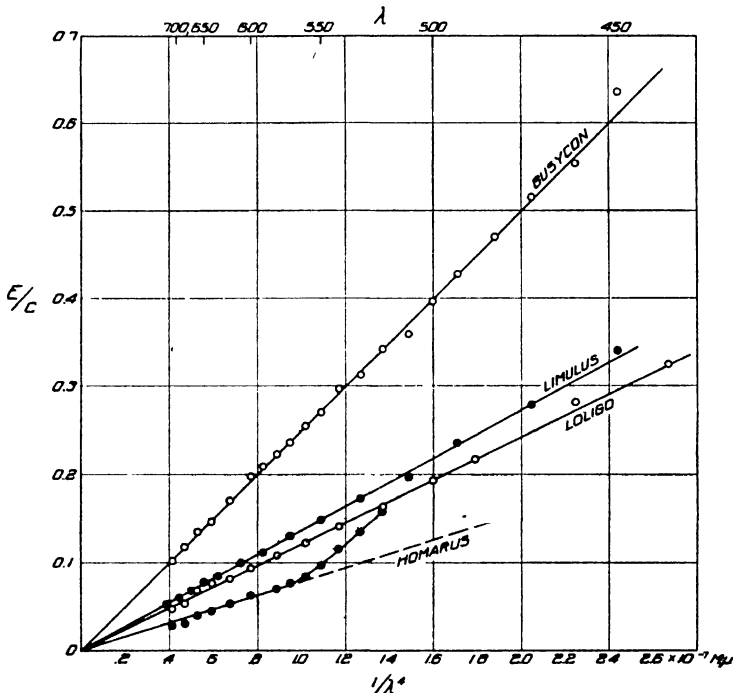


Fig. 3. Extinction coefficients, E/c , of reduced blood plotted against the reciprocal of the fourth power of the wave-length, $1/\lambda^4$.

squid and lobster. Purified preparations of reduced haemocyanins dissolved in water are almost water-clear and colourless and appear to scatter very little light, except at reactions in the neighbourhood of the isoelectric point. The scattering is greatly increased by the presence of electrolytes, and particularly by magnesium and calcium (Dhéré and Burdel, 1919; Redfield, 1930a; Redfield and Ingalls, 1932). Under these conditions, however, the scattering is diminished by the presence of alkali and, in the case of *Busycon* haemocyanin, the effect of the addition of sodium chloride disappears at reactions more alkaline than pH 8.

The interpretation of the phenomena of scattering is open to some doubt. Dhéré's suggestion that it is due to reflection of light from the large colloidal

particles is not strictly tenable, since such reflection does not vary with the reciprocal of the fourth power of the wave-length (Burton, 1921). The relation deduced by Rayleigh requires that the particles be *small* compared to the wave-length. Mecklenberg has shown that the relation holds for sulphur particles when the diameter of the particle falls between 5 and $93\text{ m}\mu$. Since the radius of the molecules of the haemocyanins of *Helix* and *Limulus* are of the order of $10\text{ m}\mu$, according to Svedberg and Heyroth (1929a), we may expect the Rayleigh equation to apply in their case. While it has frequently been assumed that the intensity of the Tyndall phenomenon depends upon the size of the particles, recent studies have indicated that the scattering of light from liquids is to be accounted for by local fluctuations in density due to thermal agitation, rather than by considerations of the size of the dissolved particles. From such considerations Raman (1927) has developed a theory of scattering by colloidal solutions in accordance with which it appears possible to relate the observed optical phenomena to the osmotic pressure of the solutions, as controlled by the equilibrium between electrolytes and protein. Until this possibility is examined quantitatively it is improper to draw inferences concerning the size of the particles of haemocyanin, or their degree of aggregation from the phenomenon of scattering.

(4) *The spectrum of the chromatic group.*

If it be assumed that the amount of scattering is the same in oxygenated as in reduced solutions of haemocyanin, it is possible to correct the absorption spectrum of the oxygenated solution for the scattering and thus obtain a measure of the absorption of light by the chromatic group formed by the union of oxygen with the portion of the molecule containing copper. Fig. 4 shows the absorption curves of oxygenated and reduced blood of *Limulus*. The intermediate curve, obtained by subtracting the ordinates of the curve for reduced blood from those of the oxygenated blood at each wave-length, represents the corrected spectrum of the chromatic group. By this method the haemocyanins of different species may be compared. Fig. 5 shows the spectrum of the chromatic groups of four species. These spectra display a considerable similarity, indicating a close chemical relationship. There exist, however, definite differences in the spectra of each species. The spectra of the chromatic group of a given species vary very little, if at all, as the result of alterations in hydrogen-ion concentration and salt content, and the differences in the spectra of different species persist after the process of purification.

The species compared by Redfield (1930a) represent widely separated groups of animals. Measurements of the absorption of light in the visible part of the spectrum by the blood pigments of the gastropods have shown that the haemocyanins of *Helix*, *Paludina*, and *Littorina* are distinctly different with regard to the chemical constitution of the active group of the molecule (Svedberg and Hedenius, 1933). In *Helix* the maximum absorption of light by the oxyhaemocyanin occurs at about $550\text{ m}\mu$; in *Paludina* at $600\text{ m}\mu$.

(5) Absorption in the ultra-violet.

Dhéré (1920) found that the haemocyanin of a number of molluscs and crustaceans showed two bands of absorption in the ultra-violet. The band with axis at $278m\mu$ was attributed to the protein portion of the molecule, being common to other proteins such as serum albumen, egg albumen, and serum globulin. The band having an axis at $346m\mu$ was considered by Dhéré to be a specific characteristic of haemocyanin and was attributed to the prosthetic group. He believed that this

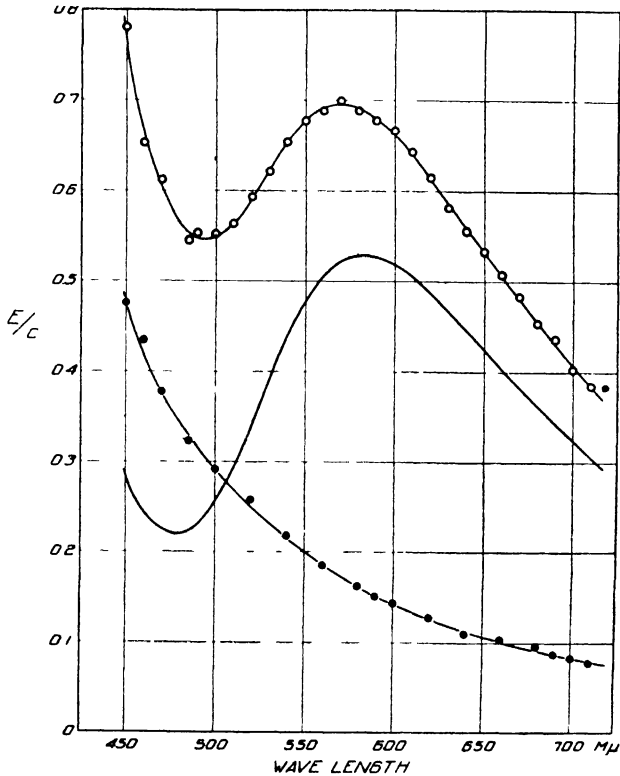


Fig. 4. Absorption spectra of blood of *Limulus polyphemus*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group.

group corresponded to the γ band of oxyhaemoglobin, which may be assigned to the porphyrin of the prosthetic group of this substance, since a band in the same region characterises the spectrum of haematoporphyrin. Roche and Dubouloz (1933) find that the band at $346m\mu$ disappears when the haemocyanin is reduced, and is replaced by progressive absorption between 380 and $300m\mu$. The ultra-violet absorption bands of oxyhaemocyanin are clearly demonstrated in the spectrophotometric curves published by Svedberg and Heyroth (1929a) for the haemocyanins of *Helix* and *Limulus*. The extinction coefficient for unit concentration is lower in *Limulus* than in *Helix*.

(6) *Beer's law.*

Quagliariello (1922) and Svedberg and Heyroth (1929*a*) present evidence that the extinction coefficient of haemocyanin solutions is not proportional to the concentration of haemocyanin. Redfield (1930*a*) showed that for solutions of haemocyanin of *Busycon* and *Limulus* Beer's law held accurately in the visible region for a range of concentrations from 0.2 to 5.5 per cent. The observations of Svedberg and Heyroth were made in the ultra-violet and for concentrations ranging

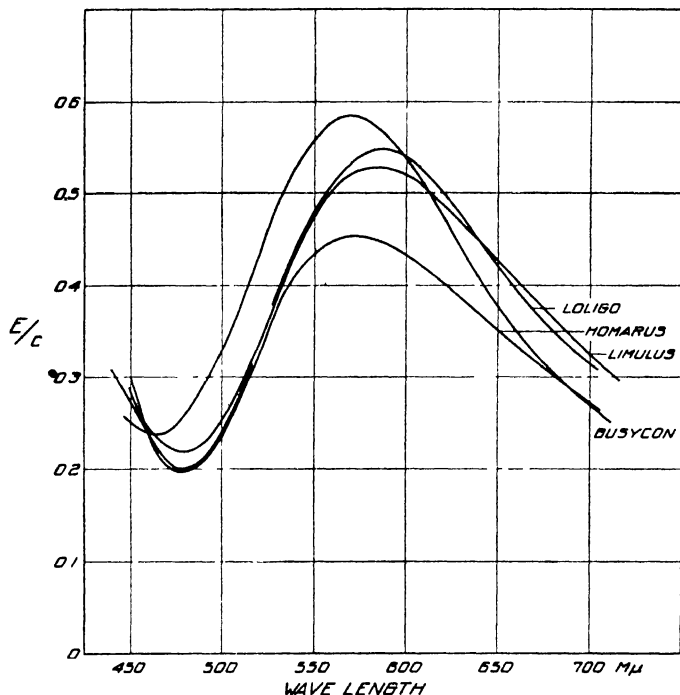


Fig. 5. Absorption spectra of chromatic groups of haemocyanin in blood of various species. Ordinate, E/c , measures extinction coefficient per milligram atom of copper per litre of blood.

from 0.136 to 0.030 per cent. The change in the light absorption for unit concentration was associated with alterations in the size of the protein units. It seems probable that the failure of Beer's law under these circumstances is due to alterations in the scattering effect, rather than to changes in true light absorption.

IV. THE PROPERTIES OF THE PROSTHETIC GROUP.

The properties of haemocyanin which have been reviewed so far result for the most part from the fact that the haemocyanins are proteins. From the physiological point of view these properties are not without interest. As globulins their characteristic solubility in salt solutions fits them for circulation in the body fluids; as

bodies of large molecular size they are readily retained within the walls of the fluid spaces in which they circulate; as ampholytes they serve as buffers in the blood, take part in the transport of carbon dioxide, and influence the equilibrium of electrolytes across the membranes bathed by the blood (Thomas, 1929). These properties are not uniquely characteristic of the haemocyanins, however, being shared by a large number of other proteins. It is the prosthetic group which enables haemocyanin to unite with oxygen and gives it its significance in respiration.

The copper complex isolated by Conant and Humphrey (1930) in their study of the prosthetic group of *Limulus* haemocyanin was insoluble in aqueous solutions. It tended to form soluble complexes with amines and denatured albumen. They suggest that the function of the prosthetic group, like that of protoporphyrin in haemoglobin, is to provide a basis for a stable metallic complex, capable of combining with oxygen. By its union with protein, this complex is rendered soluble in the blood.

(1) *Oxygenation.*

Dhéré (1900, 1903, 1915) was the first to examine the proportionality between the amount of copper present in haemocyanin solutions and the quantity of oxygen with which they would combine. It is now well established through the investigations of Begemann (1924), Redfield, Coolidge and Montgomery (1928), Guillemet and Gosselin (1932), who have examined the haemocyanin from thirteen species of animals, that one molecule of oxygen is combined with a quantity of haemocyanin containing exactly two atoms of copper. This ratio differs from that characterising haemoglobin, in which one molecule of oxygen corresponds to one atom of iron, and haemerythrin in which one molecule of oxygen appears to correspond to three atoms of iron (Florkin, 1933).

(2) *The equilibrium of haemocyanin with oxygen.*

The equilibrium between haemocyanin and oxygen has been studied in some detail by Begemann (1924), Pantin and Hogben (1925), Wolvekamp (1932), and by Hogben, Stedman, and the author and their collaborators. The oxygen dissociation curves of bloods containing haemocyanin are similar in a general way to those of bloods containing haemoglobin, and as a first approximation many of these curves can be described by the equation proposed by Hill to express the equilibrium between haemoglobin and oxygen (Hogben, 1926; Stedman and Stedman, 1926*a*; Redfield, Coolidge and Hurd, 1926). As in the case of haemoglobin, the equilibrium is influenced by hydrogen-ion activity; but between haemoglobin and haemocyanin and between the various species of haemocyanin there are important quantitative differences in this regard. Speaking generally, it may be stated that in the case of the Crustacea (*Homarus*, *Maia*, *Palinurus*, *Cancer*) and the cephalopod, *Loligo*, increasing acidity decreases the stability of oxyhaemocyanin. This effect is analogous to that first observed by Bohr, Hasselbach, and Krogh in the case of haemoglobin. The magnitude of the effect is much greater, however, for at acid reactions ($\text{pH } 6$) several hundred millimetres of oxygen

pressure may be necessary to produce half-saturation of the solution with oxygen (Fig. 6). At greater acidities also the stability of oxyhaemocyanin increases, and the "Bohr effect" is reversed, at least in the case of the Crustacea (Fig. 7). The haemocyanins which occur in the blood of *Limulus* and of the gastropods, *Helix* and *Busycon*, on the other hand, never require high oxygen pressures to produce

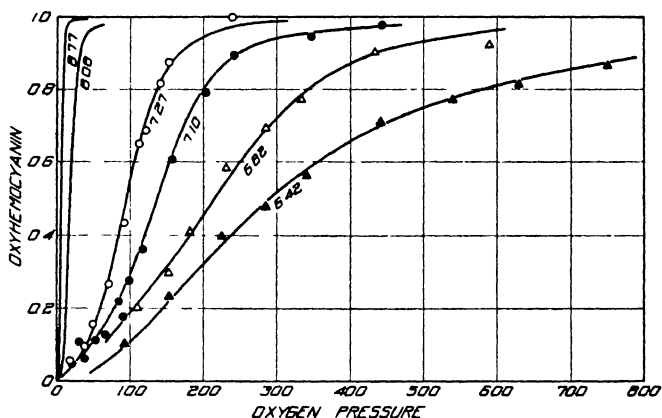


Fig. 6. Oxygen dissociation curves of blood of the lobster, *Homarus americanus*, at alkaline reactions.

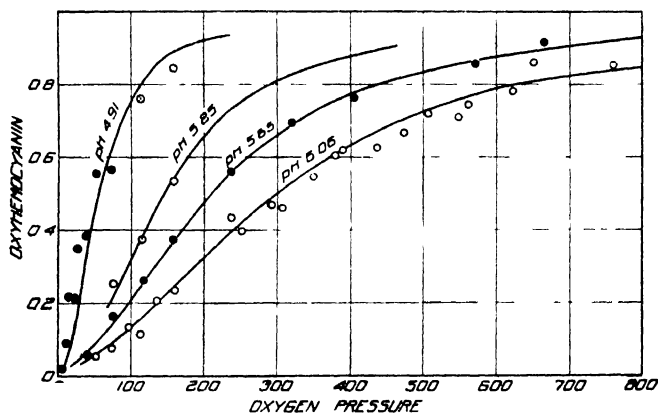


Fig. 7. Oxygen dissociation curves of blood of the lobster, *Homarus americanus*, at acid reactions.

half-saturation. In the physiological range of hydrogen-ion activities the Bohr effect is reversed, the stability of the oxyhaemocyanin increasing as the solutions become more acid (Fig. 8). At more alkaline reactions (pH 8.3 and beyond) the stability also increases so that under these conditions the Bohr effect is like that displayed by haemoglobin (Fig. 9).

The oxygen equilibrium of purified haemocyanin. In the case of the haemocyanin of *Helix*, purified by dialysis (Stedman and Stedman, 1928), and that of *Limulus* and *Busycon*, purified by precipitation at the isoelectric point (Redfield, 1930b;

Redfield and Ingalls, 1932), the equilibrium with oxygen may be described by the mass law, on the assumption that the various oxygen-combining groups react independently of one another in their combination with oxygen. The reaction may thus be written:

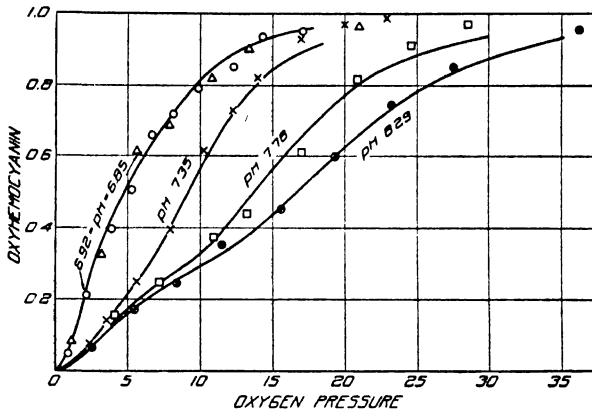
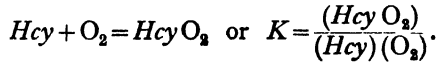


Fig. 8. Oxygen dissociation curves of blood of *Limulus polyphemus* between pH 6.85 and 8.29.

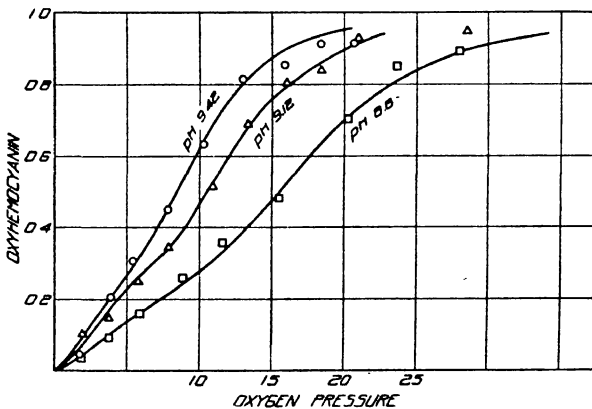


Fig. 9. Oxygen dissociation curves of blood of *Limulus polyphemus* between pH 8.6 and 9.42.

Fig. 10 illustrates this relation in the case of *Limulus* haemocyanin, the curve being drawn through the points in accordance with the above equation. This finding is similar to that made by Barcroft and Roberts (1909) in the case of purified haemoglobin, an observation which subsequent workers have found difficult to confirm. It is of interest in showing that the mass law may be applied to the oxygenation of respiratory proteins and in indicating that the more complicated dissociation curves displayed by haemocyanin in native blood are to be explained by some

modification of the mass law. The simplicity of the relationship favours the use of these materials in studying the effect of various factors on the equilibrium.

Stedman and Stedman (1928) detected no change in the value of the oxygen dissociation constant of *Helix* haemocyanin at pH values ranging from 4.04 to 9.02. The equilibrium constant of *Limulus* haemocyanin decreases as the pH value increases from 4.5 to 10.4 (Redfield, 1930b). A recalculation of the Stedmans' data suggested that a small change in the value of the oxygen equilibrium constant may occur in the case of *Helix* also.

The oxygen dissociation constant of purified *Busycon* haemocyanin was found to change quite abruptly between pH 8 and 9 (Redfield and Ingalls, 1932). This result was explained on the assumption that at this reaction a salt is formed by some acid- or base-binding group of the protein, and that this salt has an affinity for oxygen different from that of the unneutralised substance.

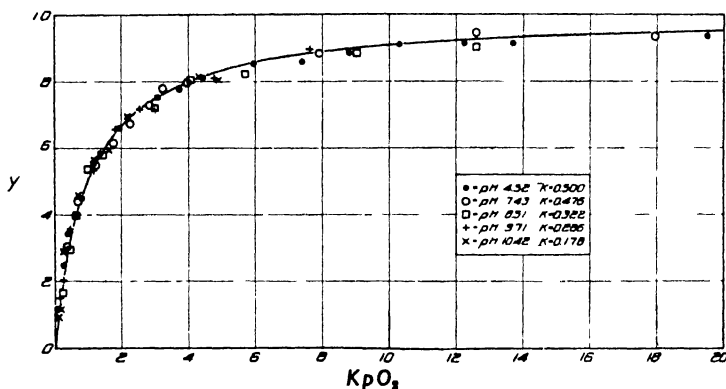


Fig. 10. Oxygen dissociation curve of purified haemocyanin of *Limulus polyphemus*. Ordinate, y , the fraction of haemocyanin present as oxyhaemocyanin. Abcissa is the product of the oxygen pressure, pO_2 , and the oxygen equilibrium constant, K . The curve is drawn in accordance with the mass law.

The effect of adding electrolytes to purified haemocyanin solutions was examined by Hogben and Pinhey (1926, 1927). While these experiments did not lead to definite conclusions, they indicated that the presence of electrolytes might alter the affinity of haemocyanin for oxygen. In the case of *Busycon* haemocyanin Redfield and Ingalls found that the form of the oxygen dissociation curve was unchanged by the addition of sodium, potassium, or magnesium chlorides, or potassium phosphate to an ionic strength of 0.5. Calcium chloride appeared to alter the shape of the oxygen dissociation curve¹.

The addition of 0.5M NaCl to purified *Busycon* haemocyanin solutions does not change the value of the oxygen equilibrium constant at acid or the more alkaline reactions. It does, however, lower the pH value at which the oxygen

¹ I have also found that the addition of sea water to purified *Limulus* haemocyanin causes the curve to become distinctly sigmoid in shape. The curve does not coincide with that of the haemocyanin in native blood at the same hydrogen-ion concentration.

equilibrium constant changes by about one pH unit. It appears that electrolytes alter the affinity of haemocyanin for oxygen principally by their effect on the dissociation constant of an acidic or basic group associated with the prosthetic group rather than directly through an effect upon the oxygen equilibrium constant.

The effect of temperature upon the equilibrium of haemocyanin with oxygen has been studied in the case of a number of bloods, both in the native and dialysed condition, by Hogben (1926), Hogben and Pinhey (1926, 1927), and Redfield and Ingalls (1933). In all cases the affinity of haemocyanin for oxygen decreases with increasing temperature. The oxygenation of haemocyanin, like haemoglobin, may be considered to be an exothermic reaction. In these cases the form of the oxygen dissociation curve is so complicated that one cannot estimate the heat of reaction without making complicating assumptions to explain the form of the curve itself. In the case of the purified haemocyanins of *Limulus* and *Busycon*, which yield dissociation curves in conformity to the mass law, the oxygen equilibrium constant is arrived at simply and the effect of temperature on its value may be easily interpreted. Brown (1933) has shown that the heat evolved in the oxygenation of these haemocyanins is approximately 14,800 calories per gram-molecule of oxygen for *Limulus* haemocyanin and 16,000 calories for *Busycon* haemocyanin.

The oxygen equilibrium of haemocyanin in native bloods. The oxygen dissociation curves of haemocyanin as it exists in the blood, and also those of the dialysed haemocyanin of the Crustacea (Stedman and Stedman, 1926*a, b*), do not have the simple hyperbolic form required by the mass law, if it is supposed that the oxygen-binding groups of the haemocyanin are situated upon separate molecules or are so separated in position on a common molecule that their reactions are independent of one another. The departure of these curves from the hyperbolic form indicates some sort of interdependence of associated prosthetic groups.

In the case of haemoglobin this interdependence has been explained by the assumption that the haemoglobin molecule consisted of aggregates of several oxygen-binding units so constituted that the oxygenated form was only stable when all of the associated groups were combined with oxygen (Hill, 1910) or that the oxygenation of the four prosthetic groups of the haemoglobin molecule takes place as a series of steps, each characterised by a distinct oxygen dissociation constant (Adair, 1925*a, b*).

By a development of the former view it is possible to account for the form of all of the oxygen dissociation curves of bloods containing haemocyanin which have been accurately described (Redfield and Ingalls, 1933). Under certain conditions of temperature and hydrogen-ion activity, the oxygen dissociation curves of the lobster and the squid are precisely described by the equation developed by Hill on the assumption that the prosthetic groups are associated in groups of two; under other conditions the data are fitted by curves calculated on the assumption that they are associated in groups of four. According to Hill's theory the fraction of the respiratory protein in the oxygenated condition, y , at any oxygen pressure, x , is given by the expression:

$$y = \frac{Kx^n}{1 + Kx^n},$$

where K is the oxygen equilibrium constant and n is the number of prosthetic groups so associated as to form a stable oxygenation product. There is thus evidence that haemocyanin may exist in forms characterised by values of $n = 1, 2$, and 4 .

The haemocyanins in the blood of the squid and lobster, under conditions intermediate between those under which n equals 2 or 4, yield oxygen dissociation curves which cannot be fitted by Hill's equation using any constant value of n . This is true under practically all conditions of the haemocyanins of the blood of *Limulus* and *Busycon*, the curves of which may have a distinctly undulatory character (Figs. 8, 9, 11). These curves may be described, however, by assuming the simultaneous presence of two or more forms of haemocyanin, each characterised by a different integral value of n . The most general form of the expression defining this assumption is:

$$y = \frac{\alpha_1 K_1 x^1}{1 + K_1 x^1} + \frac{\alpha_2 K_2 x^2}{1 + K_2 x^2} + \frac{\alpha_3 K_3 x^3}{1 + K_3 x^3} + \frac{\alpha_4 K_4 x^4}{1 + K_4 x^4}, \text{ etc.,}$$

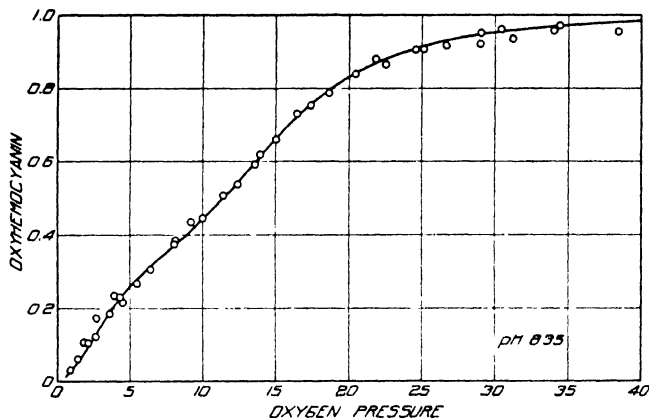


Fig. 11. Oxygen dissociation curve of haemocyanin in blood of *Busycon canaliculatum*.

where K_1, K_2, K_3, K_4 , etc., are the oxygen equilibrium constants of the forms of haemocyanin characterised by values of $n = 1, 2, 3, 4$, etc., and $\alpha_1, \alpha_2, \alpha_3, \alpha_4$, etc., are the fraction of the total oxygen bound by each of these forms.

In most cases it is not necessary to assume the simultaneous presence of more than two of the forms described by each term of the equation and practically it is never necessary to postulate the presence of a form characterised by values of n equal to three or greater than four, so that the expression used in analysing the curves may be simplified and does not require the use of terms which cannot be shown to apply alone under appropriate conditions to certain haemocyanins.

When the oxygen dissociation curves of various haemocyanins are examined systematically under varying conditions of hydrogen-ion activity and temperature and analysed in accordance with the above equation, it is found that the constants resulting from the analysis vary in a systematic manner. The values of α_1, α_2 , and α_4 indicate that in passing from acid to alkaline reactions the state of the haemocyanin

changes from the form characterised by a low value of n to one characterised by a higher value of n . At more alkaline reactions (pH 8·3 or greater) a reverse change

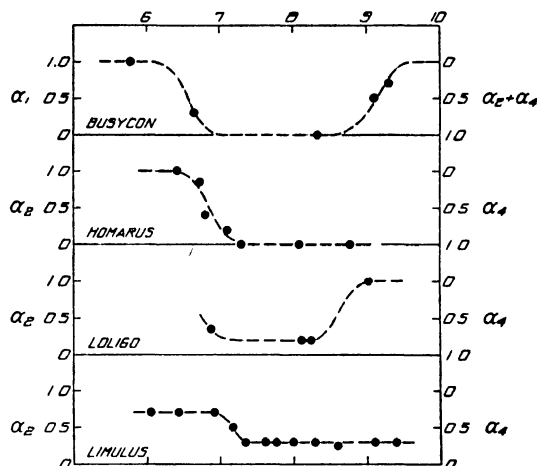


Fig. 12. Variation with pH of values of α characterising the haemocyanin of different species.

occurs in the case of the bloods of *Busycon* and *Loligo*. The values of the oxygen equilibrium constants K_1 , K_2 , and K_4 tend to decrease, indicating decreased stability

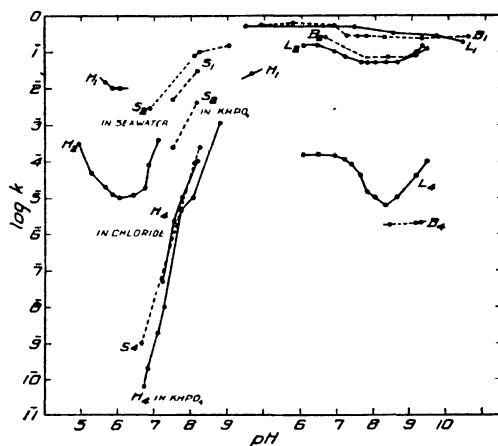


Fig. 13. Variation with pH of values of K characterising the haemocyanin of different species. H_1 , H_2 , H_4 indicate values of K_1 , K_2 , K_4 for *Homarus* blood. S_1 , S_2 , S_4 indicate values of K_1 , K_2 , K_4 for squid blood. B_1 , B_2 , B_4 indicate values for *Busycon* blood. L_1 , L_2 , L_4 indicate values for *Limulus* blood.

of the oxyhaemocyanin with decreasing acidity. After passing through a minimum, at a reaction characteristic of each species, the oxygen equilibrium constants increase again. These changes in the values of α and K , characterising various species, are summarised in Figs. 12 and 13.

It is noteworthy that the oxygen equilibrium constants characterising the haemocyanins of the squid and the lobster, on the one hand, are very similar in magnitude and in the way they vary with pH , and that those of *Limulus* and *Busycon*, on the other hand, have a similar resemblance, though differing markedly from the former species. The similarities and differences in the two groups suggest that some difference in structure of the prosthetic group of the haemocyanins of the squid and the lobster distinguishes these proteins from the haemocyanins of *Busycon* and *Limulus*.

Increasing temperature appears to change the haemocyanins of *Limulus* and the lobster from forms characterised by low values of n to forms characterised by higher values of n . The various oxygen equilibrium constants, K_1 , K_2 , and K_4 decrease in value with rising temperature as required if the oxygenation is an exothermic reaction.

(3) *The kinetics of oxygenation.*

G. Millikan (1933) has studied the rate at which haemocyanin becomes oxygenated or reduced, employing a continuous flow apparatus similar in principle to that used by Hartridge and Roughton in studying the oxygenation of haemoglobin. He finds that these reactions proceed with great rapidity. *Limulus* serum is dissociated from half its oxygen in 0.1 sec., and the serum of the crab, *Maia squinado*, in about 0.04 sec. These haemocyanins become half-saturated with oxygen in less than 0.003 sec. These velocities correspond in order to those observed for haemoglobin by Hartridge and Roughton (1925). Clearly these reactions are too rapid to limit the animals' activity appreciably.

The kinetic experiments provide independent support for the application of the theory of mass action to the oxygenation of haemocyanin. The rates of oxygen combination and oxygen dissociation were measured separately for dialysed *Limulus* haemocyanin. From these measurements the equilibrium constant for the haemocyanin was calculated and found to agree with that obtained by Redfield (1930*b*) from equilibrium studies.

Different haemocyanins were observed to differ greatly both in the speed of their reaction with oxygen and also in the way these speeds are affected by changes in hydrogen-ion activity. The haemocyanin of *Limulus* serum shows relatively little change in the rate of oxygen dissociation on altering the pH , while that of *Maia* increases its rate of oxygen dissociation tenfold as the acidity is increased from pH 9 to pH 4.

(4) *Methaemocyanin.*

The union of oxygen with haemocyanin is not an oxidation in the electronic sense, but is analogous to the change of haemoglobin to oxyhaemoglobin to which Conant (1923) has applied the term *oxygenation*. The copper present in both the reduced and oxygenated haemocyanin is in the cuprous state. It may be oxidised by sufficiently strong oxidising reagents, such as molybdcyanide or permanganate, forming compounds analogous to methaemoglobin which may be called methaemo-

cyanins. According to Conant, Chow and Schoenbach (1933), the oxidation-reduction potential of the system *Limulus* haemocyanin-methaemocyanin is approximately +0.55 volt. The change involves one hydrogen equivalent for each atom of copper in the haemocyanin. Reiss and Vellinger (1929) have measured the electrode potentials of the haemocyanin of *Palinurus* as it is oxidised, and find the reaction is half complete at a potential of +0.13 volt.

Conant, Chow and Schoenbach find that, unlike methaemoglobin, methaemocyanin retains the power of combining oxygen reversibly (oxygenation) so that oxymethaemocyanins may be formed. It is for this reason that ferricyanide cannot be used to drive off the oxygen from oxyhaemocyanin in analytical procedures, as Cook (1928) observed, and accounts in part for the fact that early attempts to demonstrate the formation of methaemocyanin have been unsuccessful (Quagliariello, 1922).

(5) *The reaction of the prosthetic group with substances other than oxygen.*

Carbon monoxide. Craifaleanu (1919*b*) observed that when carbon monoxide is bubbled through haemocyanin solutions they become colourless. Since such solutions recolour more slowly when again exposed to oxygen than is the case if the haemocyanin is reduced with an inert gas, he concluded that probably a carbon monoxide-haemocyanin compound was formed which is unstable in the presence of oxygen. Dhéré and Schneider (1922*a*) could see no difference between the action of carbon monoxide and the inert gases such as nitrogen; however, they hesitated to conclude that carbon monoxide formed no compound with haemocyanin. They pointed out that if such a compound were formed it must be colourless and less stable than oxyhaemocyanin.

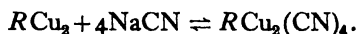
In recent experiments Root (1934) has shown that carbon monoxide combines with the haemocyanin of *Limulus* in proportions similar to those of oxygen. The compound is indistinguishable from reduced haemocyanin in colour. The affinity of haemocyanin for carbon monoxide is only one-twentieth as great as for oxygen.

Hydrocarbons. Dhéré and Schneider (1922*b*) find no evidence that the haemocyanin of *Helix* forms coloured compounds with methane, ethylene, or acetylene as claimed by Griffiths.

Nitrogen dioxide. Dhéré and Schneider (1919, 1920, 1922*b*) have shown that the haemocyanins of *Helix*, *Octopus*, and *Eledone* form a green compound on treatment with NO_2 which has been isolated in crystalline form. The nitrogen dioxide-haemocyanin is much more stable than oxyhaemocyanin. The haemocyanin of *Astacus* appears to form this compound with difficulty and that of the lobster not at all.

Cyanide. Kobert (1903) observed that haemocyanin treated with KCN becomes colourless. Craifaleanu (1919*b*) pointed out the analogy of this phenomenon to the behaviour of other complex copper salts, such as the green albuminate of copper and copper ammonium salts which are likewise decolorised by the addition of cyanide. The cyanide forms a compound with haemocyanin called cyanhaemo-

cyanin. This compound does not combine with oxygen (Cook, 1928). The reaction forms the basis for methods of estimating the oxygen combined with haemocyanin by means of the Van Slyke analysis (Redfield, Coolidge and Montgomery, 1928; Stedman and Stedman, 1926*b*). Craifaleanu found that solutions of *Octopus* cyanhaemocyanin which did not contain an excess of cyanide became blue again if exposed to oxygen, and concluded that cyanhaemocyanin is an unstable compound tending to change into oxyhaemocyanin in the presence of oxygen. Experiments made by Dr Izquierdo and Mr Pearson in the author's laboratory have failed to demonstrate any simple stoichiometric relation between the quantity of cyanide present and the loss of colour or oxygen capacity by the solutions. The results suggest that the equilibrium between cyanide and haemocyanin is a reversible one such as that represented by the following equation, in which RCu_2 represents a quantity of haemocyanin combining with one molecule of oxygen:



Hydrogen sulphide. Craifaleanu (1919*b*) found that hydrogen sulphide decomposes haemocyanin. A portion of the protein is precipitated and copper is set free and precipitated as copper sulphide.

Acids and bases. The large number of acid- and base-binding groups present in the protein portion of the haemocyanin molecule make it difficult to demonstrate that the prosthetic group has acidic or basic qualities. There is, however, some indirect evidence that this is the case.

Carbon dioxide behaves like the inert gases in reducing oxyhaemocyanin (Dh  r   and Schneider, 1922*a*). If it forms a compound with the prosthetic group, this compound is colourless. The presence of carbon dioxide and other acids, which alter the hydrogen-ion concentration of the solutions, influences the affinity of haemocyanin for oxygen, and as in the case of haemoglobin, it has been assumed that this action is due to the formation of salts by acid- or base-binding groups composing a part of the prosthetic group or closely associated with it (Redfield and Ingalls, 1932, 1933). The fact that the form of the oxygen dissociation curve of certain haemocyanins varies with the salts in which the haemocyanin is dissolved may be due to a reaction between the ions of the solvent and the prosthetic group of the protein.

A particularly interesting case in this connection is the effect of hydrochloric acid on the purified haemocyanin of *Limulus*. The haemocyanin reacts with hydrochloric acid to form a colourless compound, unable to combine with oxygen. This compound may be separated from a partially acidified haemocyanin solution by the addition of a strong solution of sodium chloride. The equilibrium between hydrochloric acid, haemocyanin, and the resulting colourless component may be described by an equation derived on the assumption that the latter is a salt formed as the result of a reaction in which the haemocyanin behaves as a divalent acid or base. The reaction is half complete at $pH\ 3.7$ and is complete upon the addition of approximately 1.0×10^{-5} mols HCl per gram of haemocyanin. From comparison with the acid titration curve of haemocyanin, which is half complete at $pH\ 3.3$ and

requires 160×10^{-5} mol per gram for completion, Redfield, Mason and Ingalls (1932) concluded that this reaction involves only a limited portion of the total basic groups, perhaps only those in the radical immediately associated with oxygen transport.

V. SPECIFICITY AND NOMENCLATURE.

There can be no doubt that the haemocyanins of the four principal groups, cephalopods, gastropods, Crustacea, and Xiphosura, are distinctly different in their chemical properties. To what extent these proteins are the same in related species is as yet uncertain, but it seems clear that smaller differences separate the haemocyanins of species belonging to the same class (Stedman and Stedman, 1926*a, b*, 1927; Hogben, 1926; Svedberg and Hedenius, 1933). As in the case of haemoglobin, the most clear-cut differences seem to be attributable to the properties of the protein portion of the molecule. Knowledge of the chemical structure of the prosthetic group of haemocyanin is still too limited to justify any statement regarding its similarity or difference in different groups of animals, though there can be no doubt that important differences do characterise its function in binding oxygen in different species. As pointed out above, there is some evidence that the haemocyanins may be divided into two groups having oxygen equilibrium constants varying in a characteristic way with hydrogen-ion activity, and these characteristics may depend on minor differences in the structure of the prosthetic group.

There is a tendency among workers in natural products to apply new names to any substance which can be shown to have any degree of individuality. In the case of such substances as haemocyanin, which appear to be characterised by different properties in almost every species examined, the nomenclature is apt to become needlessly complicated unless based on thorough chemical knowledge. For this reason I have disregarded the terms *haemosycotypin* proposed by Mendel and Bradley for the haemocyanin of *Busycon*, and *l*-, *o*-, and *h*-haemocyanin employed by Svedberg and his collaborators for the haemocyanins of *Limulus*, *Octopus*, and *Helix*. Until the various haemocyanins can be given a rational chemical classification, it seems less likely to lead to confusion if the term "haemocyanin" be reserved for all proteins having a copper complex as a prosthetic group, and if the haemocyanins of the various animals be distinguished by the scientific name of the species in which they occur.

VI. PHYSIOLOGICAL CONSIDERATIONS.

The physiological function of haemocyanin was definitely established in the case of the cephalopods by Bert (1867), who observed the change in colour of the blood, indicative of the oxygenation of the haemocyanin, as the blood passed through the gills. Analyses of the oxygen and carbon-dioxide content of the arterial and venous blood of the octopus (Winterstein, 1909) and squid (Redfield and Goodkind, 1929) demonstrate the magnitude of the change in these constituents and indicate that a very large proportion of the oxygen combined with the haemocyanin is utilised at each circuit of the circulation. Comparable observations do

not appear to have been made on other groups of animals, in which the circulatory apparatus is less conveniently arranged for physiological observation.

Certain inferences of physiological interest may be drawn from the chemical properties of the blood. The concentration of haemocyanin in the blood, and the amount of oxygen bound per gram of haemocyanin determine the total amount of oxygen which the blood may carry. The measurements of the oxygen capacity of various bloods recorded in Table VI show that in no case is the oxygen capacity of the blood as great as that obtaining in many animals possessing haemoglobin. Presumably the fact that haemocyanin occurs in solution rather than corpuscles limits, through considerations involving solubility and viscosity, the concentration of haemocyanin which may be circulated effectively.

Table VI shows that the copper content of the blood of various animals varies between about 2–20 mg. per 100 c.c. Sea water contains approximately 10 mg. of copper per cubic metre (Atkins, 1932). The copper in the blood represents a concentration over that of sea water of some 10,000-fold. Löhner (1924) has found that the fresh-water pulmonates, *Physa fontinalis* and *Lymnaea peregra*, which he considers to possess haemocyanin, are sensitive to poisoning by minute amounts of copper just as are other animals which are not provided with respiratory proteins which contain copper.

The bloods having the highest oxygen capacity are found in the cephalopods, which are also the most active of the large marine invertebrates. A similar correlation may be drawn between the activity of the animals and the pressures of oxygen required to saturate the blood. The bloods of *Limulus* and the gastropods are saturated at low oxygen pressures, as judged from the dissociation curves, and thus fit these animals for life under poor conditions of aeration, but limit the activity by maintaining the oxygen pressure of the blood at a low level during the period when oxygen is being given up to the tissues. In contrast the blood of the squid is oxygenated only at relatively high pressures and as a result the squid is extremely sensitive to oxygen lack, but is capable of superb activity when the conditions are favourable for the oxygenation of the blood. The crustaceans occupy a somewhat intermediate position. While their haemocyanin resembles that of the squid in many ways, its concentration is too low to provide a very adequate accessory to violent activity. The high oxygen pressures required to saturate the haemocyanin, especially in the presence of carbon dioxide or other acids, makes animals of this class very sensitive to asphyxiation.

The Bohr effect has received much attention in connection with the respiration of vertebrates. It is recognised that the effect of carbon dioxide in turning out oxygen from the blood in the tissues and the reciprocal effect in the lungs are responsible for a significant part of the mammalian respiratory exchange. In the case of the squid, in which the Bohr effect is large, it is estimated that one-third of the respiratory exchange results from this phenomenon (Redfield and Goodkind, 1929). The conditions leading to asphyxiation are determined by the properties of the oxygen dissociation curve as influenced by carbon dioxide, death occurring when the oxygen and carbon-dioxide pressures are such that the arterial blood can

Table VI. Oxygen capacity, copper content, and haemocyanin content of blood of various animals. The oxygen capacity represents the amount of oxygen present when the blood is equilibrated with air. The copper content is calculated from this value assuming 0.49 volume per cent. to be dissolved and two atoms of copper present per molecule of oxygen combined. The haemocyanin concentration is calculated from the composition shown in Table I for the same species.

Species	Oxygen capacity vol. %	Copper mg. per 100 c.c.	Haemo-cyanin %	Authority
Cephalopods				
<i>Octopus vulgaris</i>	3.1-4.5	1.48-22.8	5.9-9.1	Henze, 1901; Dhéré, 1903; Winterstein, 1909; Craifaleanu, 1919a
<i>Loligo pealei</i>	3.8-4.5	18.8-22.8	7.2-8.8	Redfield, Coolidge and Hurd, 1926
Gastropods				
<i>Helix pomatia</i>	1.15-2.2	3.75-9.70	1.47-3.9	Cuénot, 1891; Dhéré, 1900; Begemann, 1924
<i>Helix aspersa</i>	1.2	4.03	—	Cuénot, 1900-1
<i>Busycon canaliculatum</i>	2.1-3.35	9.16-16.2	3.7-6.6	Redfield, Coolidge and Hurd, 1926
<i>B. carica</i>	1.36	4.94	—	Redfield, Coolidge and Montgomery, 1928
Crustacea				
<i>Astacus fluviatilis</i>	2.4	10.8	—	Dhéré, 1900
<i>Homarus vulgaris</i>	3.1	—	—	Dhéré, 1900
<i>H. vulgaris</i>	1.22	4.14-14.8	—	Stedman and Stedman, 1925
<i>H. americanus</i>	1.95	8.3	4.4	Redfield, Coolidge and Montgomery, 1928
<i>Palinurus vulgaris</i>	1.43-1.80	5.34-7.45	—	Winterstein, 1909; Stedman and Stedman, 1925
<i>Cancer pagurus</i>	1.6-2.3	6.31-10.3	—	Dhéré, 1903; Stedman and Stedman, 1925
<i>C. irroratus</i>	1.23-1.69	4.2-6.82	—	Redfield, Coolidge and Hurd, 1926
<i>C. borealis</i>	1.40	5.16	—	Redfield, Coolidge and Montgomery, 1928
<i>Carcinus maenas</i>	1.14-1.16	3.69-3.80	—	Begemann, 1924
<i>Callinectes sapidus</i>	1.29	4.54	—	Redfield, Coolidge and Hurd, 1926
<i>Ovalipes ocellatus</i>	1.78	7.33	—	Redfield, Coolidge and Montgomery, 1928
<i>Maia squinado</i>	0.84-1.75	1.99-7.16	—	Winterstein, 1909; Stedman and Stedman, 1925
Xiphosura				
<i>Limulus polyphemus</i>	0.74-2.7	1.42-12.6	0.8-7.3	Jolyet, 1895; Redfield, Coolidge and Hurd, 1926

combine only 0.5 to 1.5 volumes per cent. oxygen. Recent experiments by Dr Eric Fries indicate that the oxygen pressures at which asphyxiation occurs at various temperatures may also be correlated with the effect of temperature on the oxygen equilibrium in the blood.

In the gastropods and *Limulus* the Bohr effect is reversed under physiological conditions. It may be argued that this property of the blood favours the absorption of oxygen at the gill. The ease with which the phenomenon may be shown to be

an advantage, irrespective of which way the system works, makes one very sceptical of the teleological argument.

The buffering of bloods containing haemocyanin does not appear to show any unique phenomena. Buffer action results from the protein nature of the respiratory pigments and is in a general way dependent on the concentration of these substances. Bloods containing haemocyanin are not inferior to other bloods of like oxygen capacity in buffer action, and inasmuch as buffering under normal conditions is concerned with the transport of carbon dioxide, there is no need for caring for an amount of carbon dioxide greater than that equivalent to the oxygen transport. Gram for gram haemocyanin is not an inferior buffer to haemoglobin, as the values cited on p. 187 show. It has been pointed out, in discussing the effect of oxygenation upon the titration curve of haemocyanin, that the change in the dissociation constant of the acid-combining group of haemoglobin which is effected on oxygenation falls within the series showing the magnitude of this phenomenon among the haemocyanins.

In conclusion it may be pointed out that while the quantitative differences which distinguish the various haemocyanins have made the comparative study of these compounds fertile from a chemical point of view, the bloods containing haemocyanin do not form as interesting an evolutionary series as do those provided with haemoglobin (Redfield, 1933). This is perhaps due to the fact that evolution among the haemocyanin-bearers has resulted in a great variety of forms, but has led to little advance in the mechanisms for respiration, whereas the series of animals provided with haemoglobin has culminated in the production of animals capable of intense activity in a terrestrial and aerial environment.

VII. SUMMARY.

The haemocyanins are proteins combined with a prosthetic group consisting of a complex copper salt of a sulphur compound and a polypeptide. The physical and chemical properties of haemocyanin are largely due to its protein nature, and the specific differences between the haemocyanins of different groups of animals result largely from the differences in the protein part of the molecule. The combination of haemocyanin with oxygen and the characteristic change in the absorption spectrum which results are, on the other hand, properties of the prosthetic group. The haemocyanin combines with oxygen in all cases in proportion to its copper content, one molecule of oxygen being combined by a quantity of haemocyanin containing two atoms of copper. There is some evidence that at least two modifications of the prosthetic group may occur characterising the Crustacea and cephalopods on the one hand, and the gastropods and *Limulus* on the other. The equilibrium with oxygen may be described by the mass law if it be assumed that under different conditions one, two, or four prosthetic groups must be oxygenated simultaneously in order to form a stable compound and that under certain conditions a mixture of such forms of the protein exist. In general the oxygenation phenomena displayed by haemocyanin differ from those of haemoglobin only in a quantitative sense.

The copper of the prosthetic group is in the cuprous condition but may be oxidised, forming methaemocyanin, by strong agents. Methaemocyanin will combine reversibly with oxygen, forming oxymethaemocyanin. Haemocyanin also combines with carbon monoxide, nitrogen dioxide and with cyanide, forming stable compounds.

The physiological function of haemocyanin as a respiratory pigment is well established in the case of the cephalopods. Some correlation exists between the activity of various animals, the oxygen capacity of the blood, and the pressures of oxygen under which the haemocyanin becomes oxygenated.

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THE BIRD FAUNA OF THE GALAPAGOS ISLANDS IN RELATION TO SPECIES FORMATION

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CONTENTS.

	PAGE
I. Introduction	213
II. Physical features of the Galapagos Islands and some previous studies of their fauna	214
III. Origins of the avifauna	215
IV. Mode of arrival	220
V. Non-resident species and migration routes	223
VI. Trends of variation	223
VII. Summary	233
References	234

I. INTRODUCTION.

SINCE their first discovery by the scientific world, the Galapagos Islands in their fauna and flora have presented a wealth of subject-matter for study and speculation. Similar material scrutinised by different individuals has supplied bases for widely different conclusions, and this itself has been a spur toward the acquisition of further data. In some groups, as in birds and reptiles, large collections have been assembled, sufficient, or nearly so, for such classification as is concerned with definition of the finer divisions. Besides the accumulation of actual specimens, field work upon the islands and advancing knowledge of other regions that must be considered in the same connection are making it possible to draw comparisons and deductions, to point out analogies, and to come to at least a few conclusions with a degree of finality that was not possible some years ago. After several years devoted to a systematic study of the birds, based upon abundant museum material, I was able to spend two months in the summer of 1932 in field observations that included most of the Galapagos Islands, an experience so stimulating and enlightening as regards problems presented in the avifauna as to emphasise anew my conviction of the overwhelming importance of such an approach. Opinions expressed in the following pages are, consciously or unconsciously, the reflection of impressions conveyed by the living birds and their native surroundings.

II. PHYSICAL FEATURES OF THE GALAPAGOS ISLANDS AND SOME PREVIOUS STUDIES OF THEIR FAUNA.

The Galapagos Archipelago, comprising nine larger islands and a number of smaller ones, lies on the Equator in the Pacific Ocean about 500 miles west of the coast of Ecuador, and a slightly greater distance south-west of Panama. The islands are of volcanic origin, and volcanic activity continues to the present day on the two westernmost islands. Equatorial heat is appreciably modified by the cold Humboldt current sweeping northward, especially on the western side of the archipelago, and at sea level there is little in the plant growth that is suggestive of the tropics. There are wet and dry belts, mainly altitudinal, with rain falling mostly on the summits and southern exposures of the mountains; and there are wet and dry seasons of the year, though these periods are very irregular both as to dates and amount of precipitation. In some sections there are large areas of lava flow, barren of vegetation or nearly so, but for the most part the islands have a dense growth of shrubbery. At low elevations this consists of a conspicuously large proportion of cactus, thickets of mesquite and other plants of similar habit, with mangrove along many of the shores; higher up, in the rain belt, there is a jungle of larger trees, and on the higher summits many square miles that are grass grown or covered with large ferns. The islands present widely different aspects seasonally, due largely to the host of annuals that springs up with the rains, to wither away in the dry heat of the rainless period. The Galapagos are almost destitute of fresh water, the porous lava absorbing the rainfall, so that any surface run-off is of the most temporary nature. One small stream on Chatham Island is perhaps the only permanent flow. Springs of fresh water are very few and of trifling volume, and there are only two or three fresh-water lakes, and those of small size.

The Galapagos support an abundant fauna, in which birds are conspicuous, and the outstanding peculiarities of these birds, together with the striking manner in which evolutionary processes are illustrated thereby, have again and again attracted the attention of the philosophical biologist.

Charles Darwin was the first naturalist to visit the group, and the inspiration he derived from the bird life, with the far-reaching effect of the deductions he made therefrom, are matters of history. Darwin, from geological studies, regarded the Galapagos as oceanic islands. The feature of the animal life that most impressed him, a new idea in that age, was that, with the individuals of a species varying more or less in minor respects upon different islands, the fauna as a whole was obviously "created on American types of organisation." The particular part of America from which it was derived, on which later studies have been concentrated, did not then, of course, assume any particular importance.

The next to pursue critical studies of the avifauna of the Galapagos was Salvin (1876), who further demonstrated the American origin of the birds, and who upheld Darwin's conclusions as to the oceanic character of the islands. Years later came the important work of Dr George Baur, who collected his material in 1891. A systematic report upon his birds was written by Robert Ridgway (1897), who

apparently concurred in the accepted view of the islands' origin, but Baur himself wrote a series of papers (1891, 1897) presenting strong arguments, based mostly upon the bird life, in demonstration of a former continental connection of the Galapagos toward the Panamic region. Later students of ornithology have not agreed with Baur's views, but Van Denburgh (1912, 1914), from investigation of the abundant and extraordinary reptile fauna, arrived at the same conclusion. As a result of a recent study of the birds (1931), it is my belief that the animal life of the Galapagos arrived there fortuitously after emergence of the islands from the sea, not as a result of former continental connection.

Study of species formation as illustrated in the Galapagos avifauna divides itself under three heads: place of origin, mode of arrival, and the observed results of insular isolation.

III. ORIGINS OF THE AVIFAUNA.

As regards the origin of the Galapagos birds, whether the islands are continental or oceanic we can definitely put aside the supposition of former connection with, or accessibility to, the adjacent mainland of South America. If such affiliation had existed there must have been surviving upon the islands to-day some representation of the abundant and highly characteristic avifauna of Ecuador and Peru. The only Galapagos bird which occurs in those countries is the Cuckoo, and that is found also to the northward in Colombia, whence it is more likely to have arrived. A possible former union or approach must be looked for in the direction of Panama or Costa Rica, which lie not so much farther to the north-east than Ecuador does to the east.

The Galapagos avifauna is such as might be expected to occur on a group of oceanic islands; its character argues against a former continental connection. There are certain marine species whose occurrence here, hinging on factors controlling such species the world over, has no bearing upon the former accessibility or connection of the Galapagos toward other regions. There is an important element clearly recognisable as of West Indian affinities, that could have had no other derivation. There are other species that might have come from either a West Indian or a Central American source, and there are a few species that definitely do not belong to the West Indian avifauna. There is not one species that can be recognised as having necessarily come from the adjacent South American coast. There are a number of species so widely differentiated as to make their immediate derivation and relationships impossible of recognition. The avifauna as a whole is extraordinary in its segregation and its strongly developed characteristics. Only two of the long list of resident land birds occur elsewhere, one on the South American mainland, one upon Cocos Island.

It seems now a reasonable hypothesis to place the inception of the Galapagos avifauna in a period when North and South America were separated by the sea. The Galapagos Islands, Cocos Island and Malpelo Island could then be regarded as distant outliers of the West Indian Archipelago, and relationship of the faunas

of these two, now separated, areas (West Indies and Galapagos) could be explained on the same basis as relationship from one to another of the West Indian islands.

Examination of the list of Galapagos birds with regard to general distribution of each species and its immediate relatives, together with the mode of variation, will demonstrate the basis of the above generalisations regarding the source or sources of the avifauna of the islands. A preliminary division into water birds and land birds is useless, for water birds are not necessarily marine and may in this case be subject to exactly the same inhibiting factors as land birds.

First on the list is the Galapagos Penguin, the one unmistakable immigrant from the far south, though the Flightless Cormorant may well be of southern

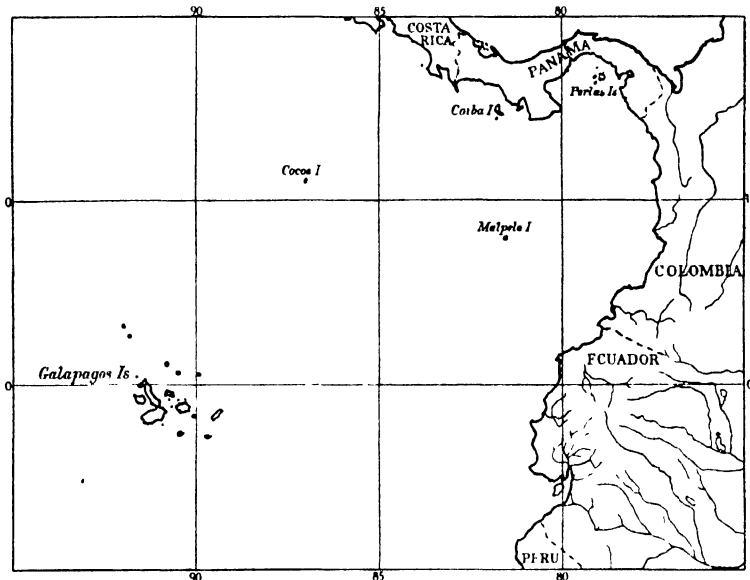


Fig. 1. The Galapagos Archipelago, in relation to the coast of Ecuador to the east, and Panama to the north-east.

origin, too. Then come the truly marine species, Albatross, Petrels, Boobies, and Tropic-bird, the presence of which is probably the result of quite different causes from those affecting the more sedentary fresh-water and terrestrial species. The peculiar Galapagos Albatross, for example, is doubtless to be accounted for on the same basis as the other species of that family elsewhere, each confined to an island or a restricted archipelago. Specific differentiation in the Albatrosses becomes explicable in the extraordinary devotion shown to one limited breeding area, in the Galapagos species to one particular island. Even among the sea birds, though, there are doubtless some whose establishment here is the result of former West Indian association, others of Pacific origin that may or may not have arrived at a later date.

In the Petrels, Boobies and Tropic-bird the origins are not so readily traced.

The two species of Frigate-bird are much more satisfactory. It seems clear that *Fregata magnificens* remains as a member of the ancient West Indian avifauna, and that *F. minor ridgwayi* arrived here and on the west coast of Mexico, the easternmost limit of its range, at a period when conditions forbade any farther advance. The uprising Central American land-mass first divided the *magnificens* population in two; later it barred the new coming *minor* population from further progress.

The herons raise certain questions that are more easily suggested than answered. First, the family Ardeidae, with four distinct genera and species, is unusually well represented upon the Galapagos. Then, the four species offer wide differences in the mode and extent of differentiation from their nearest relatives. The American

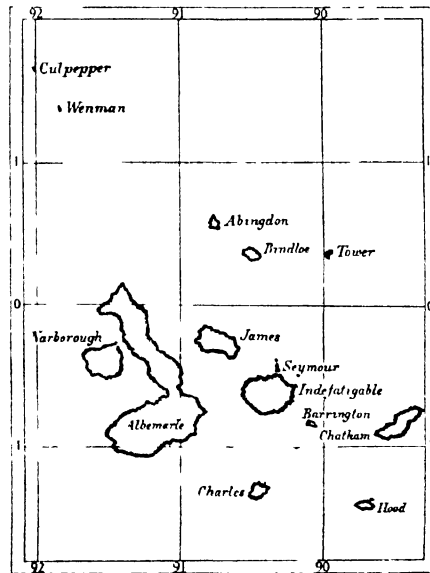


Fig. 2. The principal islands of the Galapagos Archipelago.

Egret occurs throughout tropical and part of temperate America; it is apparently unchanged upon these islands. Pure white in colour, it is only in mensural characters that there is opportunity for variation. The few specimens available hardly suffice to show minor variations in this respect. The Great Blue Heron is North American and West Indian (of dubious occurrence even in extreme northern South America); the Galapagos population is slightly but distinguishably characterised. The Yellow-crowned Night Heron, of the warmer parts of America, is again slightly differentiated in a Galapagos variety. The Galapagos Green Heron, member of a genus that occurs around the world, is too strongly differentiated from its American relatives for its appearance to afford any certain clue to its immediate origin. There are distinguishable local strains upon certain of the islands.

Did these four kinds of herons, variously differentiated as they are from their several ancestral stocks, arrive upon the Galapagos at the same time? It might be

so, despite surface appearances to the contrary. The extraordinary Galapagos Green Heron is, of course, the difficult part of the problem. The Great Blue Heron (*Ardea herodias*) and the North American Green Heron (*Butorides virescens*) are of widespread distribution over North America and the West Indies, occupying almost the same ground. They are both "plastic" species, within certain bounds, exhibiting slight variation in different regions and to about the same degree. *Ardea herodias* upon the Galapagos has developed a "sub-species" that is comparable to its North American variants. The Green Herons (*Butorides*) have representation as far as Cocos Island in the West Indian sub-species *Butorides virescens maculatus*; on the Galapagos in the abruptly and strikingly different species *B. sundevalli*.

In the absence of evidence testifying to the sources of these several birds as being from different regions and at different periods, there is the need for scrutinising any known facts that might bear upon the apparently inconsistent present-day assemblage of Galapagos herons. On the island of Cuba there exists, together with the common Green Heron of the region, another form of great rarity, "*B. brunescens*" (Lembeye). This has been regarded by different authorities variously as a distinct species and as a colour phase of *B. virescens*. Whatever the truth of its taxonomy, at any rate there are in Cuba two distinct "kinds" of Green Heron upon the same ground. It is conceivable that colonisation of the Galapagos was from the stock of a comparable variant of the past, under circumstances that permitted the isolation and subsequent development of certain characters that were peculiar to this variant alone. The presence of "*brunescens*" in Cuba shows that *Butorides*, however rarely, is capable of producing an aberrant offshoot in a new direction. Perhaps such a variant was ancestral to the Galapagos *sundevalli*, disappearing later in its original home. Some such explanation seems to me to be demanded, in the absence of any fact suggesting the arrival of *Butorides* upon the Galapagos from some direction other than the West Indies. The occurrence of the West Indian *B. virescens maculatus* in the Pacific on Cocos Island should be borne in mind.

Incidentally, adaptation to a markedly littoral habitat, as is seen in the several Galapagos herons, might be cited as an example of results when a chance controlled wanderer, arriving on a distant island, is obliged and able to exist in an environment that would not be its normal choice. In the absence of fresh-water lakes, streams and marshes, these herons have successfully turned to tide pools, reefs, and rock-strewn shore lines.

The Brown Pelican (*Pelecanus occidentalis*), Frigate-bird (*Fregata magnificens*), Flamingo (*Phoenicopterus ruber*), Galapagos Pintail (*Paecilonitta galapagoensis*), and Mangrove Warbler (*Dendroica petechia aureola*) are unmistakably of West Indian origin. The Pelican and the Flamingo of the two regions are indistinguishable; the Frigate-bird and the Pintail exhibit slight differences; and the Mangrove Warbler likewise shows slight ("sub-specific") variation but occurs in the same variety upon the Galapagos and Cocos Island. Study of the general distribution of each of these species points to their occurrence on the Galapagos as a result of former, more free, communication between those islands and the West Indies, so

conclusively it seems to me as to be beyond further argument. There are other species, such as the several herons, Gallinule, and Stilt, that might very well be similarly derived.

Of birds that are clearly not of Antillean affinities the most conspicuous are the several forms of the Vermilion Flycatcher (*Pyrocephalus*). The genus ranges from extreme south-western United States southward over most of tropical America, but (except upon the Galapagos) it is confined to the mainland. It is, curiously, absent from most of Panama. The Galapagos forms, two well-differentiated species, are too unlike their mainland relatives to afford a clue to their origin in their appearance, but there is one bit of negative evidence. On the nearby South American mainland, in western Peru, the local species of *Pyrocephalus* exhibits a dusky phase, so distinct as to have been described as a separate form, and the Galapagos birds show no trace of this peculiarity. Presumably, therefore, they arrived from some more northern region—Mexico or Central America.

The Cuckoo (*Coccyzus melacoryphus*) is the one resident land bird that occurs unchanged upon the Galapagos and on the South American mainland. As it is a Colombian species it may be supposed to have arrived from the direction of that country rather than from the adjacent west coast of South America, an assumption that is justified only in the entire lack of evidence that any other Galapagos bird whatsoever did arrive from the west coast. Cuckoos are not common upon the islands, and they are shy as compared with their associates, these traits, together with the unchanged appearance of island birds, suggesting relatively recent arrival. Upon Cocos Island there is a sharply distinct species of Cuckoo (*Coccyzus ferrugineus*) confined to that one island. The Galapagos Oyster-catcher (*Haematopus palliatus galapagensis*) is another bird that is not of Atlantic origin. It is hardly to be distinguished from the Lower Californian sub-species, *H. p. frazari*. Perhaps both are to be explained as immigrants from farther south.

There is a long list of Galapagos birds so strikingly differentiated that their appearance no longer affords information as to their immediate derivation and relationships; at least my own experience and knowledge do not suffice to recognise the clue. These include the following: Hawk (*Buteo galapagoensis*), Black Rail (*Creciscus spilonotus*), Swallow-tailed Gull (*Creagrus furcatus*), Sooty Gull (*Larus fuliginosus*), Dove (*Nesopelia galapagoensis*), Barn Owl (*Tyto punctatissima*), Short-eared Owl (*Asio galapagoensis*), Crested Flycatcher (*Myarchus magnirostris*), Purple Martin (*Progne modesta*), Mockingbirds (*Nesomimus*), and the Geospizidae. The Galapagos Hawk bears a superficial resemblance to *Buteo swainsoni* of western North America, and likewise belongs to the group having three "notched" primaries. The large hawk that has colonised the islands off the coast of Mexico is the very different *B. borealis*. The Galapagos Black Rail is closely similar to *Creciscus jamaicensis* of North America and the West Indies; there are other rails throughout South America that are regarded by at least one modern systematist as sub-species of *C. jamaicensis*. The family of Mockingbirds (Mimidae) reaches its greatest development in Middle America and the West Indies. It is poorly represented in South America, and it is prone to develop distinct forms on island

habitats. The Galapagos genus *Nesomimus* in its varied specific and sub-specific manifestations was apparently developed upon the archipelago from a single ancestral form, and one that probably came from the north-east. On the Ecuadorean mainland there is only one species of Mimidae, of the widespread genus *Mimus*.

IV. MODE OF ARRIVAL.

Among students of birds, Baur is the outstanding advocate of the theory of a former connection with the mainland, in his opinion toward Central America and the West Indies. His conviction is based primarily upon the "harmonic" nature of the avifauna, namely, that genera and certain outstanding species (the major groups) are of widespread distribution, while species of a genus, and sub-species of a species, are more closely restricted to one or a few islands. The orderly mode of occurrence and variation of slightly differentiated forms that is seen in the Galapagos is pointed out by Baur as impossible of attainment through the accidental arrival of species upon a group of oceanic islands. Actually, this harmonic appearance is derived from the overwhelming numbers of a few groups, the omnipresent Geospizidae (the so-called "Finches"), with thirty-seven species and sub-species, the Mockingbirds (*Nesomimus*), with ten species and sub-species, and the Vermilion Flycatchers (*Pyrocephalus*), with three forms. The conviction that these groups were developed upon the Galapagos, each from a single ancestral immigrant form, gives a different aspect to the picture, which then is seen as a harmonious development that took place upon the islands. This is a different thing from a representative section of a harmonic continental fauna suddenly isolated.

Rothschild and Hartert (1899), arguing against continental connection, remark: "It is doubtless, in our opinion, quite as intelligible, that the various islands have been populated from one island, where an ancestral form was living. Thus, they were reached at various times, and by-and-by, through isolation, the separated colonies became slightly changed, without the necessity of assuming a submergence of a great area, the existence of which is opposed to geological observations and theories."

Van Denburgh (1912, 1914), and Van Denburgh and Slevin (1913), working with reptiles, are committed to the submergence theory, of the lowering of continental connection first, of intra-Galapagos connections later. Translation of mode and amount of reptilian variation inspires confident assertions of the relative time and progress of submergence in different parts of the archipelago. It seems to me that in their manner of representation, that is, a few major forms (tortoise, lizard, gecko, iguana, and snake) developed into local varieties of varying numbers, the reptiles present a condition very similar to that in the birds. There are required five ancestral immigrant forms established upon the Galapagos in the first place; surely with former continental connection toward tropical America more of the abundant reptile fauna of the mainland would be represented among the islands to-day.

As regards distribution and variation among the different islands of the archipelago, local emergence or submergence may have had a part, but I have not been

able to synchronise conditions among birds with those ascribed to reptiles. Birds, of course, have power of locomotion far beyond reptiles, but, even so, there are many bird species in the Galapagos that actually are closely delimited within certain boundaries that their wings permit them to pass at will. There is probably not a land-bird species in the Galapagos that would not be able to colonise the entire archipelago, flying from island to island, but they do not do so. It seems to me fair to compare, with caution, conditions between birds and reptiles.

My own feeling regarding the birds is that with a former continental connection, either east or north, we should see a different sort of avifauna upon the Galapagos to-day. The present bird population, though extremely abundant as regards individuals, is, as regards representation of different groups, of just the sparse and miscellaneous character that might be expected to result from the occasional arrival of chance-controlled immigrants. Had there been former connection with the mainland of tropical America it is inconceivable that there should not have been retained some representation of such dominant groups as the Parrots, Woodpeckers, Hummingbirds and Antbirds, to mention a few. Turning to other groups than birds, we note the absence of amphibians and the scanty representation of land mammals (comprising only one bat and five species of a group of small-sized rodents), giving evidence against a former continental connection. At the same time, the complexion of the avifauna that did establish itself shows plainly enough that, whatever the manner of arrival, at some period circumstances rendered the Galapagos relatively easy of approach for non-marine birds from a West Indian-Central American source, an approach that never has been open from the South American coast.

The presence of a breeding colony of the Sooty Tern (*Sterna fuscata crissalis*) upon Culpepper, the northernmost island of the group, affords an instructive commentary upon the frequent obscurity of factors governing the distribution of species. This strong-flying bird has bridged the wide gap between the Sooty Tern metropolis off the coast of Mexico and the north end of the Galapagos, but for no obvious reason it has not taken the additional easy step toward the unlimited nesting grounds afforded by the other islands. Occasional individuals stray to the southward but that is all. The rare occurrence off the northern Galapagos of the White Tern (*Gygis alba*), which nests commonly upon Cocos Island, is perhaps of similar significance. It is possible that in some manner the presence of these birds is dependent upon the warm Panama current, which sweeps southward about this far. However the place was reached, and for how long a period occupied as a nesting ground, it should be noted that there is here an outlying southern colony of a Middle American bird; and that there is not on the Galapagos any outlying western colony of a single one of the sea-bird species that swarm along the South American mainland coast.

There are certain bird genera and species that have demonstrated a surprising ability to colonise remote and widely separated islands the world over, and on the Galapagos we recognise in this category the Gallinule (*Gallinula chloropus cachinnans*), the Black-necked Stilt (*Himantopus mexicanus*), the Barn Owl (*Tyto punctatissima*),

and the Short-eared Owl (*Asio galapagoensis*). We can appreciate the reasonable probability of these birds reaching this distant objective if the way was open for any species at all; but it is not easy to recognise the conditions that permitted the passage of four species of herons, including the Yellow-crowned Night Heron, and that barred the Black-crowned Night Heron (*Nycticorax*), which is of surprisingly widespread and insular distribution. A parallel case is afforded by the presence of the Gallinule (*Gallinula*) on the Galapagos, to the exclusion of the Purple Gallinule (*Ionornis*) and the Coot (*Fulica*). The absence of *Fulica* from the Galapagos is remarkable, considering the wide distribution and varied development of the genus throughout temperate and tropical America; *Gallinula* and *Fulica* both occur on the Hawaiian Islands.

That the absence of certain groups is as much the result of chance as the presence of others is borne out by the existence of types of habitat ("ecological niches") that are unoccupied. As an outstanding example there may be cited the lack of woodpeckers and other birds of similar habits. Besides the abundant forest trees, there are in the lowlands magnificent groves of giant cactus of several species. On the mainland these latter plants have attracted a varied assemblage of birds, their occupation of the cactus made possible through the presence of certain peculiar woodpeckers whose labours supply nesting sites for all. On the Galapagos, forest trees and cactus are still unoccupied by woodpeckers. Potential followers of the woodpeckers are there, at least in the Purple Martin and Crested Flycatcher, but obliged to seek other nesting sites and probably handicapped accordingly. Then, the Rock Wrens and Cañon Wrens of North and Middle America and many of their Pacific islands would find there ideal habitats of vast extent that are unoccupied by any species. No, I do not think that it can be argued that the number of forms on the Galapagos are to the number on the mainland about in just proportion to the varieties of habitat. There are unquestionably upon the Galapagos ecological niches perfectly adapted to certain specialised mainland species, but now as always beyond the reach of their potential occupants.

Cocos Island (north-east of the Galapagos and about midway toward Costa Rica) and Malpelo Island (a barren rock about midway between the Galapagos and Panama) both have their parts in a study of the source of the Galapagos fauna. The tiny Cocos Island possesses only four species of land birds, but these four are of striking character. They are the Cuckoo (*Coccyzus ferrugineus*), Flycatcher (*Nesotriccus ridgwayi*), Mangrove Warbler (*Dendroica petechia aureola*) and "Finch" (*Pinaroloxias inornata*). The Cuckoo and Flycatcher, both peculiar to Cocos, are too sharply differentiated to permit recognition of their immediate affinities. The Finch, also restricted to Cocos, is recognisable as a member of the Geospizidae, the only species known to occur elsewhere than in the Galapagos. The Mangrove Warbler in the sub-species *aureola* occurs on Cocos and the Galapagos, nowhere else. On Malpelo Island there is apparently a small colony of the Swallow-tailed Gull, otherwise restricted closely to the Galapagos.

V. NON-RESIDENT SPECIES AND MIGRATION ROUTES.

The Galapagos are visited regularly by a number of migrants from the north. So far there have been recorded one duck (the Blue-winged Teal), the Osprey, fifteen species of wading birds, Barn, Cliff and Bank Swallows, and Bobolink. The seasonal migration of birds appears to form a problem quite separate and apart from the slow shifting and adaptation of the breeding habitat, continued through the ages. Many of the northern waders remain upon the Galapagos through the year, but never to breed, a common occurrence with such birds in other parts of the world. The breeding range of a species appears never to be suddenly extended through migrants remaining to nest at distant favourable localities. So the migration routes followed by the several northern visitants to the Galapagos have undoubtedly a history that is quite different from the circumstances that have established the residents thereon. Some of the waders clearly come south over the Pacific; the Bobolink certainly, the Blue-winged Teal probably—both are species of the Mississippi Valley—travel due south over Mexico on a line that, continued, brings them to the Galapagos. Other species may come either way.

As regards the long list of breeding birds, every one, I feel sure, so far as nesting activities are concerned, is absolutely restricted to the archipelago; in almost every case specific or sub-specific differences make it apparent. None of the land birds ever leave the islands. Many of the sea birds, of course, go far afield at some seasons, but even these with little doubt return unfailingly to their birthplace. The peculiar Albatross demonstrates this condition absolutely, and it probably applies to all others too.

There are, of course, curious anomalies in local distribution. The Albatross is restricted to Hood Island; the Penguin and Cormorant are each confined to the western part of the archipelago, the Cormorant within remarkably narrow limits; the Sooty Tern is restricted to the northernmost islands; the Hawk is absent from Charles Island; and there are other less striking restrictions.

VI. TRENDS OF VARIATION.

The numerous islands forming the Galapagos Archipelago cannot be divided into sections on any faunal or floral basis. So far as the birds are concerned even the very different "dry" and "wet" belts, mainly altitudinal, do not exhibit any decisive differences in their inhabitants. In some of the widespread species there are complicated series of variants over the different islands, and in some of the more stable forms there are curious peculiarities in distribution, but these are all upon a basis of specific vagaries, or else connected with ecological requirements. There are apparently no widely applicable sets of conditions that serve to segregate whole assemblages of birds within restricted limits, as is the case with plants. One island is much like another in the general complexion of the avifauna, each containing a more or less extensive representation of the same, or corresponding, sets of species.

There are enough species on the Galapagos characterised by being in what appears to be an arrested stage of plumage development to be worthy of comment. Conspicuous among these is the Red-footed Booby (*Sula piscator websteri*). Most of these birds on the Galapagos are not in the white and black adult plumage, but are in the dull, uniformly brownish garb of immaturity. The ratio of white birds to brown on the breeding grounds is at the most one to fifty; on the Mexican islands where the species also nests, the breeding birds are practically all in the adult white and black plumage. This Pacific coast sub-species, *websteri*, is distinguished from the typical form by having, even in the adult plumage, brownish grey instead of white tail feathers. The black and white pattern of normal adults over most of the range of the Red-footed Booby is in the evolution of the species presumably a later development than the uniform brown coloration of the young bird. Do the usually brown Galapagos birds, and the white but usually brown-tailed Mexican birds, illustrate stages toward the ultimate assumption of the adult white and black stage? Or, is there in the Galapagos strain an inhibition that commonly obtains against the assumption of the normal adult plumage?

The Galapagos Pintail Duck (*Paecilonitta galapagoensis*) is very slightly differentiated from the Bahaman Pintail, the only colour difference being that in the former the white cheeks merge gradually into the dark colour of the rest of the head, while in the latter there is a sharply defined line of demarcation. The Galapagos Pintail thus gives the impression of being in an arrested stage. In the Geospizidae there are some striking examples of this sort of vagary. In *Geospiza* the adult male is ordinarily black throughout, the female streaked; in the genera *Platypiza* and *Camarhynchus* the adult male is ordinarily black-headed, the female without black. But there are certain islands where the non-black condition in all three genera is the usual thing, most adult males never attaining to the black stage. This will be treated more fully below.

The statement has been made that the Galapagos avifauna as a whole shows a strong tendency toward melanism. The evidence is not conclusive but it is worth considering. Certainly, as one wanders over black lava reefs, with dusky marine iguanas under foot, the dark-coloured Galapagos Green Heron scrambling out of the way, companies of Sooty Gulls clamouring overhead, and black Finches coming and going, the whole combines toward a sombre tone that is rather impressive. Whether, however, this is all a result of environment is an unanswered question. The conspicuously abundant black or blackish Geospizids may, I think, be set aside in this connection as supplying no more convincing evidence than would the black Red-wings (*Agelaius*) if they had chanced to become established there. In either case blackness seems to be an inherent character of the group that would become evident in any surroundings.

The sooty Green Heron and the sooty Hawk, however, do give the impression of being surviving dusky strains of dimorphic species, of which the "normal-plumaged" strain has almost disappeared. They are both species of groups in which dimorphism is a common phenomenon, sometimes with a degree of geographic segregation. The Sooty Gull, too, clearly belongs to a black-headed group

of gulls, though the outline of the dusky hood is now all but lost in the generally blackish colour.

On the other hand, the Vermilion Flycatcher (*Pyrocephalus*) is worth considering in this connection. On the nearby Peruvian mainland there is a species of this genus in which a melanic phase is so strongly developed as to have been named as a distinct species. There is, thus, a tendency in this direction existent in *Pyrocephalus*. That it has not appeared in the two Galapagos species is due partly, no doubt, to their relatively remote relationship toward the Peruvian form, but it may be cited, too, as evidence against the presence of a melanic stimulus in the Galapagos environment.

There are 112 species and sub-species of birds in the Galapagos list, of which 89 breed upon the islands. Of the 89 breeding birds (divided among 26 families) the overwhelming majority are clearly differentiated from their nearest relatives. Including even the wide-ranging sea birds, I find only ten that have escaped sub-specific naming at one time or another. It is not possible to make definite lists, as some names have been applied on grounds that it has not been possible to investigate, and there are one or two species still bearing the name of the mainland form that some systematists would separate with little hesitation. But at any rate, there are only a few of the residents, like the Cuckoo and the Brown Pelican, that have thus far defied recognition of any differentiation, and there is a respectable list (Black-necked Stilt, Oyster-catcher, Great Blue Heron, and some of the sea birds) that can be arranged in a graded series showing advancing degrees of distinctness, leading to the 70 or 80 per cent. of the population that is so strikingly peculiar. There is one family, the Geospizidae, and four genera of four other families, *Nannopterum*, *Creagrus*, *Nesopelia* and *Nesomimus*, that are practically restricted to the Galapagos. (The exceptions consist in the occurrence of a Geospizid on Cocos Island, of *Creagrus* on Malpelo Island.)

The Geospizids, including the so-called Galapagos Finches, are the group that at once comes to mind when Galapagos birds are mentioned. This includes 37 named and recognisable species and sub-species (perhaps as many more synonyms), divided into five genera; an additional genus and species occupies Cocos Island. One genus (*Certhidea*) was formerly placed with the Honey Creepers (Coerebidae), then with the Wood Warblers (Mniotiltidae), but despite widely different externals *Certhidea* and *Geospiza* are demonstrably of close relationship. It seems evident that this entire assemblage was developed upon the Galapagos from a single ancestral immigrant species that later became diversified. Incidentally, it became unstable in form of bill and in other characters that are generally depended upon by taxonomists. Other variable bird species have produced different forms upon different islands, but the Geospizids are the only group of land birds with more than one form in one place. They occupy the entire archipelago, with from four to eleven species on an island. These birds in their curious variations, complicated inter-relations, and manner of occurrence, present many facts worth dwelling upon.

Variations consist in general size, bill structure and colour. They are all small birds, from warbler-size (*Certhidea*) up to the larger finches (*Geospiza*). Bill

variation is extraordinary, from the delicate, warbler-like *Certhidea* bill, through others variously starling-like, tanager-like, and finch-like, the latter type varying again from very small up to the unwieldy beak of *Geospiza magnirostris*, perhaps the heaviest structure of its sort among birds of this general size. In *Geospiza* the adult male is black, the female streaked or else dusky; in *Platyspiza* and *Camarhynchus* the adult male is ordinarily black-headed, the female is sometimes streaked, sometimes uniformly buffy or yellowish; in *Cactospiza* the sexes are alike and pale-coloured; in *Certhidea* the sexes are essentially alike and without black markings, ranging from almost pure white to pale brownish, the male in most of the species with a chestnut area on the throat. The Cocos Island *Pinaroloxias* is warbler-like in size and structure; the male is black, the female streaked. Throughout the Geospizidae, in adults of both sexes the bill changes colour seasonally, being black during the breeding periods, pale coloured at other times. In the young bird the bill is pale coloured in both sexes.

In *Geospiza* an all-black plumage, in *Platyspiza* and *Camarhynchus* a black-headed plumage, in most forms of *Certhidea* a chestnut-throated plumage, is regarded as the "perfect" or "fully mature" condition of the adult male. These plumages may be admitted to be the "perfect" stage of the adult male, but all males do not necessarily reach those stages. It is a notable fact that a different percentage of males in this "perfect" plumage should occur upon different islands, and also that several species, not closely related, should be similarly affected upon the same island. Abingdon Island affords the most striking example of this condition. There are eight forms of Geospizidae upon Abingdon (omitting *Certhidea*), in which plumage conditions are as follows in the series studied: *Geospiza magnirostris*, 21 males, of which 5 are black, 16 streaked; *G. fortis*, 22 males, 3 black, 19 streaked; *G. fuliginosa minor*, 17 males, 4 black, 13 streaked; *G. difficilis*, 6 males, 4 black, 2 streaked; *G. scandens abingdoni*, 9 males, all streaked; *Platyspiza crassirostris*, 9 males, 3 black-headed, 6 streaked; *Camarhynchus habeli*, 8 males, 1 black-headed, 7 streaked; *C. p. parvulus*, 1 streaked male.

It is thus apparent that on Abingdon Island the "perfect" plumaged males are extremely scarce in all species, with one possible exception. And it must be borne in mind that the efforts of the average collector would be directed toward securing the high-plumaged birds, so that in the actual population there is probably a lesser proportion of such than is shown in the collected series. Bindloe is close to Abingdon, and the two are nearer to each other than to any other island, yet conditions on Bindloe are very different in that high-plumaged males are in the majority. On Chatham Island, again, there is a very small proportion of high-plumaged males in some species; in others they are found in normal numbers. In contrast to those islands where the "immature" plumage preponderates, we find certain species upon Barrington, Tower, James and Jervis, with nearly all the mature males in the "perfect" plumage. Thus, while different species are similarly affected upon Abingdon, the same species is differently affected upon Abingdon and, say, Jervis. There are many variants to the situation among different islands and different species.

There are other general trends of variation. On Chatham Island there are local representatives of the *Geospiza scandens* group and the *Cactospiza pallida* group. These two species are characterised by relatively long slender bills, but the Chatham Island colony of each shows a distinct shortening and thickening of that member. *Geospiza magnirostris* (large), *G. fortis* (medium), and *G. fuliginosa* (small), represent three size stages in species that are otherwise alike, and the three occur together on most of the islands. The largest-billed *magnirostris* is on the northernmost islands, and size diminishes steadily to the southward; the species does not occur on the three large southernmost islands. In *G. fortis*, the largest-billed birds are on the southernmost islands, and size diminishes to the northward. The same is true of the diminutive *G. fuliginosa*. The three species intergrade through individual variation, and all three may be found in mixed flocks, feeding together.

In contrast to those widespread forms showing more or less variability from island to island, is the peculiar genus and species, *Platyspiza crassirostris*, which ranges practically unchanged throughout the archipelago. This species too, however, exhibits the suppression of the normal adult plumage on Abingdon Island. Then, although most of the sharply defined species of limited range are found on the small, outlying islands, there is *Geospiza debilirostris* on the large central islands, James and Indefatigable, which is curiously restricted by its ecological requirements. The actions of this bird suggest the desirability of field studies on other peculiar forms.

In distribution and manner of occurrence on the islands, it will be seen that the different forms arrange themselves in groups, and that these groups, in their different members (sub-species or closely related species), are distributed more or less widely throughout the archipelago. The avifauna of each island includes representatives of different groups, not several representatives of any one group. Thus, James Island, with eleven species, does not include any two that are very closely related; but it does have one representative of the four sub-species of *Geospiza scandens*, one representative of the three sub-species of *Cactospiza pallida*, one of the eight species of *Certhidea*, and other comparable representation. Stated another way, from the point of view of distribution of species, it may be said that *Geospiza scandens* has representative forms (sub-species) on different islands, no two on any one island; as is also the case with *Cactospiza pallida*, with *Certhidea*, and with other forms. The central islands have the greater number of species, 11 on James, 11 on Indefatigable, and 10 on Albemarle; of the outlying islands, there are 4 species on Tower, 4 on Hood, 9 on Abingdon, and 7 on Bindloe. But it should be noted that the islands with the fewest species have the greatest proportion of forms that are peculiar to them. Of the four Geospizids upon Tower Island, three are restricted thereto, of the four species upon Hood, two are distinct. It seems curious that, among the outlying islands, there should be as many "ground finches" upon far distant Culpepper, and more upon Wenman, Abingdon and Bindloe, than upon Tower and Hood, no farther from the main group, but this condition doubtless results from the same factor that has produced such sharply differentiated species among the few forms that have succeeded in reaching, or

surviving upon, the two last mentioned islands. There are 3 of these "finches" reported from Culpepper, 5 from Wenman, 8 from Abingdon, and 6 from Bindloe, as compared with 3 each from Tower and Hood. Wenman and Abingdon have been reached by stray individuals of species that have never wandered to Tower. There is some evidence of a wandering (migration, of a sort) of species from the central islands, from island to island toward the north, but not to Tower, far distant in the north-east.

The genus *Certhidea*, with eight recognisable forms, is peculiar in the difficulties presented toward any coherent grouping of species or sub-species. Island variation affects colour and pattern almost entirely; structural differences are insignificant, an extraordinary fact, considering conditions in the other genera. Variation between islands, and variation in series from any one island, is such as to suggest sub-specific treatment of the different forms, and, in fact, it would be quite possible and logical, upon the basis of overlapping through individual variation, to regard the group as a monotypic genus and to treat all of the forms of *Certhidea*, widely different as some of them are, as sub-species of one species, *C. olivacea* Gould. To do this, however, would in some instances necessitate the acceptance of intergradation between series from widely separated islands, with diverse forms interposed between, and it seems doubtful if such an arrangement would indicate in fact the actual relationships and the true manner of divergence between the several forms, as it would appear to do. Despite the strong predilection that I felt for sub-specific treatment at the outset, it is Ridgway's (1902) course, of using a binomial for each form, that I have finally adopted. As a matter of fact, the outcome of a careful weighing of pros and cons in the different possible nomenclatural methods of treatment of *Certhidea*, is an almost total abandonment on my part of any attempt at expressing relationships through names. Binomials are used simply as a means of referring to the *Certhidea* population on the several islands or aggregations of islands that are inhabited by distinguishable forms.

Snodgrass and Heller (1904), treating of the finch-like species, outline six different plumage stages which they claim represent an orderly development throughout the group and which they use as a basis for their classification. Their theory, briefly, is that the plumages of these "finches" show a progression from a primitive plain buffy-yellow colour upward through streaked and black-headed stages to an entirely black condition. The plain-coloured *Cactospiza* is placed at the bottom, and *Geospiza conirostris* (with black male and blackish female) at the top; the young of the several intermediate stages are described as reverting each to an immediately lower stage. The much more abundant material that is now at hand shows such wide departures from their proposed arrangement as to make it impossible of acceptance, at least in its entirety. Certain plain-coloured species are now known to be streaked in the juvenal plumage, and other unconforming peculiarities have been discovered in the young stages of other species as well.

The most recent classification (Swarth, 1931) recognises six genera (five on the Galapagos, one on Cocos Island), based upon colour, pattern, and bill structure. Two of these genera are monotypic, within the others there are a number of more

or less closely resembling forms. It seems desirable to regard these as species or sub-species mainly from the degree of difference and the abruptness of change. Intergradation of characters occurs to a bewildering degree, from one extreme to another, though not always between birds that are geographically adjacent. In fact, any discussion centring upon the question as to what system the classification of these birds should follow, whether a given form is a species or a sub-species, or whether or not it is a "good" sub-species, is rather beside the mark, they so resolutely refuse to conform to the standards applied to continental species. A system of names regarded as labels to so many pigeon-holes of definite capacity is out of the question; on the other hand, rigid adherence to accepted criteria for sub-specific association of forms could be followed to absurd lengths. Certain writers have lumped the genera *Geospiza*, *Platyspiza*, *Cactospiza*, and *Camarhynchus* in one genus and upon plausible grounds, but the same argument could be advanced for the inclusion of *Certhidea* as well. Furthermore, it would be just as possible to argue for the specific unity of all the forms concerned (from *Geospiza* to *Certhidea*) to regard them all as only sub-specifically separate. Intergradation through individual variation can be traced between any of the extremes, though not always between forms that are geographically adjacent. There is abundant material on hand for ordinary purposes of classification, but most assuredly the facts demonstrated thereby do not lend themselves satisfactorily to interpretation through our current system. Whether the bewildering conditions existent among these island birds arise entirely from the presence of factors that are ordinarily absent from the surroundings of mainland forms, or whether they are due in part to an instability in rapidly succeeding generations such as is not commonly seen elsewhere, cannot be said, but I incline to the latter view.

Difficulties in classification of these extraordinary birds are no more than those encountered in seeking adaptational values in the different lines of development. Snodgrass (1902), in his study of these birds, concluded that there was no correlation between food and the widely variable size and shape of bill. In other words, natural selection was eliminated as a factor in the production of the observed variations, and apparently justly so, for in the amount and sort of differentiation that is seen here, and in the extraordinary amount of intergradation, it is not apparent that there are useful adaptations in the remarkable extremes nor any lessened fitness in the numerous intermediates. There are large bills and small bills, heavy bills and slender bills, among the ground-feeding species of *Geospiza*, and also, pushed to nearly as great extremes, among the tree-frequenting genera.

There are, however, differences of habits that are fairly well correlated with *Geospiza* on the one hand, with *Platyspiza*, *Cactospiza*, and *Camarhynchus*, on the other. The species of *Geospiza* ("ground finches") are for the most part ground feeders, though the long-billed *scandens* and its allies (the "cactus finches") resort primarily to cactus (eating both fruit and blossom) and to the introduced oranges and other fruits. *Platyspiza*, *Cactospiza*, and *Camarhynchus* ("tree finches") are tree dwellers, feeding on leaves, fruit and insects in the shrubbery, rarely on the

ground. It is noticeable that it is in the more sharply differentiated species, such as *Geospiza debilirostris* (strictly terrestrial and with skulking, rail-like habits), and *Platyspiza crassirostris* (noticeably arboreal), that there is found the most rigid adherence to certain given surroundings. In the abundant, widely distributed, and widely variable species, *Geospiza fortis* and *G. fuliginosa*, food requirements are not so rigidly restricted, these birds being noted as feeding chiefly on the ground, but also commonly in trees and bushes, among the rocks on the beaches, and even picking at carrion and among the refuse of a camp. Many of the finches have turned to introduced oranges and other fruit, to such an extent, indeed, in one case (*G. scandens*), as to cause that bird to have spread in abundance into the humid zone on islands where oranges are established in that belt, while elsewhere, under primitive conditions, it is characteristic of the arid zone, dependent upon the cactus fruit.

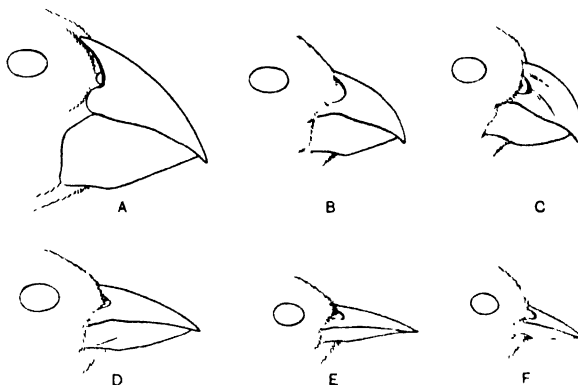


Fig. 3. Variation in bill structure in the six genera of Geospizidae. A, Great-billed Ground-finch (*Geospiza magnirostris*). B, Darwin's Tree-finch (*Platyspiza crassirostris*). C, Parrot Tree-finch (*Camarhynchus psittacula*). D, Pallid Tree-finch (*Cactospiza pallida*). E, Cocos Island Tree-finch (*Pinaroloxias inornata*). F, Darwin's Certhidea (*Certhidea olivacea*). About natural size.

As Gulick (1932) has expressed it: "The generic characters are to a considerable extent adaptations to differing stations and food habits, but the marked differences between lesser geographical races and the large individual fluctuations seem to stand in no relation to food, and not to be greatly subject to natural selection. . . .

"It would appear that these birds have contributed to science one of our finest examples of what happens when an animal has moved out of the closely competitive life on continents and become subject to an island environment that offers a great diversity of alternative opportunities, unhindered by the competition of rival species. Diversification comes to be actually at a premium. . . . and even natural selection builds up a tendency toward instability of type."

Altogether, in many of the Galapagoan Geospizidae there is seen a variability in physical characteristics and an adaptability in habits that argues well for their future. It is in striking contrast to the highly specialised development found in,

and rigid requirements controlling, the avifaunas of oceanic islands elsewhere, of which the Hawaiian birds (often compared with those of the Galapagos) at once come into mind.

The mode and amount of variation in these birds suggest that various types of development are being pushed to extremes, and without the elimination of the connecting intermediates; the extraordinary variants that crop up in many of the series give an impression of a process of change and experiment going on. Such remarkable extremes of variation in bill structure as are seen, for example, in series of *fortis* or *fuliginosa* from any one of the larger islands, connected as they are by every intermediate stage, lie outside my experience with any North American mainland bird. All these features give trouble, of course, in any attempt at an orderly classification of the forms concerned.

Darwin has stated one objection to the theory of evolution through natural selection in the following words: "Why, if species have descended from other species by insensibly fine gradations, do we not everywhere see innumerable transitional forms? Why is not all nature in confusion, instead of the species being, as we see them, well defined?" Various answers, more or less satisfactory, have been made to these queries, but here just the conditions that are predicated by Darwin are what seem to obtain among the very birds that first inspired his researches in evolutionary problems. There *are* "innumerable transitional forms" (and, also, aberrant individuals apparently departing in entirely new directions). There *is* a pronounced degree of confusion, and some forms that we are obliged to treat as species are *not* well defined.

One feature of these birds that has been a stumbling-block to orderly classification is the extraordinary number of individual variants, single specimens that in some one character, generally bill structure, depart so widely from the most nearly related form as to give a first impression of specific distinction. Such were the unique "*Geospiza dentirotis*" Gould, and "*Cactornis brevirostris*" Ridgway. This happens sufficiently often to make it seem possible that notably different variants are appearing not uncommonly among these birds, but not necessarily perpetuating their peculiarities.

Next to the Geospizids the most important bird group is the genus *Nesomimus* (Mockingbirds), peculiar to the Galapagos and distributed throughout the archipelago. There are four distinct species, three of them severally restricted, each to one large island with its nearby islets, the fourth divided into a number of recognisable sub-species and distributed over many islands. The three first mentioned occur upon three large islands at the south-eastern extremity of the archipelago, islands that are nearer to each other than to the rest of the group. Another variable genus is *Pyrocephalus* (Vermilion Flycatcher), which has developed one sharply distinguished species upon Chatham Island, a slightly variable species over the rest of the archipelago.

Then there are various other bird groups, mostly representatives of mainland genera, but nearly all distinct and sharply differentiated species, restricted to the Galapagos and showing various peculiarities of distribution. In several cases these

are divided into clearly differentiated varieties upon different islands, and in other species there can be found upon one island or another some slight departure from the mode of its kind. Examples are found in the Green Heron and the Crested Flycatchers on Chatham Island, the Hawk on Hood, the Dove on Culpepper and Wenman, and the Barn Owl, showing slight differences between Albemarle and Indefatigable. There does not seem to be any conformity in all this. On the contrary, it is suggestive of the possibility of the several species having obtained their first foothold on the Galapagos at different points, perhaps at different times, and, accordingly, producing their most strongly differentiated forms where varied circumstances governed.

The Galapagos Green Heron is one species of which the known facts can be presented in a somewhat coherent argument. This bird in its typical form is distinguished from all others of the genus by the absence in the adult of pale edgings to the wing coverts; from other American species by dark coloration and heavy bill and feet. The Chatham Island variant is paler coloured, the general pallor of the head and neck bringing out certain markings that are not visible in the ordinary dark-coloured bird, and all the wing coverts are narrowly edged with buffy white. These conditions suggest that the Galapagos Green Heron (*Butorides sundevalli*) is descended from a melanic strain in some ancestral *Butorides* immigrant, that normal *sundevalli* represents the farthest departure from the original *Butorides* characters, and that the Chatham Island birds are in an arrested, intermediate stage. The latter still possess the light-margined wing coverts, a character that they share in common with all other species of the genus; only in typical *sundevalli* from the rest of the Galapagos has this feature been lost. The apparently inconsequential facial markings of the Chatham Island birds, blackish and whitish streaks that extend backwards from eye and mandible, are also deep-seated generic characters repeated in other species. Just as the Chatham Island herons depart from average *sundevalli* in appearance, so do they approach other species of the genus. Primitive characters are retained, and there is no apparent development in new directions.

In the foregoing pages there are outlined some of the salient features of a remarkable avifauna. Many striking circumstances are only briefly indicated or entirely omitted, and in almost any direction careful scrutiny of available facts and known conditions would suggest promising lines of inquiry. Almost everyone who has seriously studied the birds has expressed the conviction that understanding was most apt to be reached through out-door investigation by someone who could devote a long period to the task. After an all too brief visit to the islands, I am of the same opinion, with the added proviso that such a student should have a background of experience with birds in other regions, the wider the better. A most important factor in the situation is the absolute indifference of all Galapagos birds to human visitors. If they cannot every one be plucked off the bushes or from the rocks—and with many of them this is possible—they can be approached within arm's reach and examined or photographed with no loss of time or energy in con-

cealing manœuvres. It seems likely that breeding experiments could be carried on with some species as readily as with the domestic fowl.

Anyway, there the birds are, together with other striking features of the fauna, presenting scientific opportunities that can hardly be duplicated elsewhere in the world. The tortoises have suffered terribly by human persecution, nearly to extinction, but the birds, with some other forms of life, have been miraculously preserved into our own time in almost their primitive condition. Some knowledge exists, of course, of how to utilise this material to ascertain information potential in such an assemblage of animal life, and it would be a tragedy if, as might easily come about, some slight change in conditions should wipe out whole sections of this fauna or make difficult or impossible the pursuit of important studies that would now be feasible. The enactment and enforcement of measures of conservation—there are none at present—with the establishment of a modest biological laboratory upon one of the islands, represent an investment in research that would promise solid returns.

VII. SUMMARY.

The Galapagos Islands possess a peculiar and highly characteristic fauna and flora. The abundant bird and reptile populations are nearly all of endemic species; of the land birds only two species occur elsewhere than in these islands. Studies bearing upon the origin of the Galapagos fauna have led to diverse conclusions; scrutiny of modes of variation has revealed some curious situations. The Galapagos have been variously regarded as the surviving remnants of a land-mass, now sunken, that was formerly connected with the American mainland, and as oceanic islands that have appeared above the ocean as the result of volcanic upheaval. Study of the birds is confirmatory of the latter view. The avifauna is clearly not derived from the South American mainland directly to the eastward. Of the marine species there are one or two of southern origin, borne northward on the cold Humboldt current, and there are others which constitute local forms of species that are of world-wide distribution. There is an important element definitely recognisable as of West Indian derivation, and others may have originated from the same source. There are a few species that clearly are not of West Indian ancestry, and there are a number that are too widely differentiated for recognition of their immediate affinities. The hypothesis is advanced that the inception of the Galapagos avifauna took place in a period when North and South America were separated by the sea; the relationship of the faunas of the West Indies and the Galapagos is to be regarded in the same light as relationships from one to another of the West Indian islands. The bird population of the Galapagos, abundant as regards individuals, is, as regards representation of different groups, of the sparse and miscellaneous character to be expected of chance-controlled wanderers to distant islands.

Conditions are uniform enough throughout the archipelago, so that, with much local variation, each island contains a fair representation of the same general assemblage of species. Trends of variation are seen in arrested stages of plumage

in certain species, in a possible tendency to melanism in others. There are many variants of these situations. The outstanding group of birds is the endemic family, the Geospizidae, including 37 species and sub-species out of the entire list of 89 breeding birds. Extensive variation and complicated relationships within this family are such as can probably not be duplicated in any mainland stock of birds. The observed variation presents difficulties to classification, and certain trends of development seem to act independently of natural selection. The Geospizidae afford a fine example of diversification unhindered by competition.

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THE PROTEOLYTIC ENZYMES OF MICRO-ORGANISMS

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CONTENTS.

	PAGE
I. Introduction	235
II. The breakdown of native proteins	238
(1) Putrefaction	238
(2) Aerobic decomposition	240
III. Bacterial enzymes	242
(1) Soluble enzymes found in the medium	242
(2) Endocellular enzymes	247
IV. The enzymes of yeast	248
(V) The enzymes of fungi and Protozoa	249
VI. The effect of the composition of the medium on the formation of enzymes	253
VII. Résumé of methods	256
VIII. The present position	257
References	259

I. INTRODUCTION.

UNDER this heading it is proposed to review the literature concerning those enzyme systems of micro-organisms which bring about decomposition either of relatively unchanged proteins such as native ovalbumin, serum albumins and globulins, or derived proteins and substances like gelatin, as well as the smaller units of the protein molecule, peptones and polypeptides. The breakdown of amino acids will not be discussed: the work on this subject requires separate treatment for adequate presentation. There is a good deal of confusion in the literature with regard to nomenclature, and the terminology adopted in this review is as follows. By proteolytic enzyme, or protease, is understood any enzyme capable of attacking a protein or its derivatives, these terms being used in quite a general way. When it is desired to speak specifically of an enzyme attacking whole proteins, Willstätter's term *proteinase* will be used. The titles polypeptidase, dipeptidase, etc., referring to enzymes attacking polypeptides and dipeptides respectively, are self-explanatory. As a rule the general term "protease" has to be used for enzymes studied prior to the last few years, as it is only within the latter period that critical separation of the components has been carried out.

A good deal of work has been done on the proteolytic enzymes of the lower organisms, but the subject has been approached from so many different angles

that few attempts have been made to view it as a whole. Further knowledge of the nature of these enzymes and particularly of the factors which influence their synthesis by the organism, apart from its own intrinsic interest, must have far-reaching effects in such diverse fields as tissue invasion and breakdown in pathological conditions, all those ripening and maturing processes in foodstuffs of microbial origin, and the large-scale transport and storage of foodstuffs so essential to the modern community.

We have at present no means of classifying enzymes except by their effects, so that any scheme adopted must be purely arbitrary, and future work may show that enzymes now placed in different groups are really the same enzyme acting under slightly different conditions. Investigations in other fields, for example the bacterial dehydrogenases, have indicated that it is better to postulate one active group located in this case at or near the cell surface, together with some such factor as specific adsorption, rather than upwards of fifty different enzymes (Quastel, 1926). A somewhat similar view is gaining ground with regard to proteolytic enzymes. Willstätter and his school have brought forward evidence for regarding them as active groups associated with protein carriers (Willstätter, 1922). Fodor (1926) concluded that with the enzyme peptidase the activity is independent of the protein carrier and the active group could in fact be transferred to kaolin or glycine. In the latter case its properties were different from those when in association with the protein. Northrop (1929), on the other hand, claims to have isolated crystalline pepsin in a pure state, the pure substance having the properties of a protein. A general criticism which is often levelled against the "surface action" idea of enzyme structure is that it does not always account for the extremely specific nature of enzyme action. On the one hand, if we postulate that every reaction catalysed is the work of a specific and definite enzyme, we have to picture how a cell which for the sake of the present argument we may call a cube of side 3μ , or a sphere of similar dimensions, containing about 70 per cent. water, can accommodate all these enzymes, remembering that the diameter of crystalloid molecules may be of the order of $1m\mu$, while colloidal particles may be of the order of $100m\mu$ in diameter. On the other hand, if we hold the view that an enzyme is an area of intense activity set up locally by polarisation or other means, we have to develop an adequate mechanism to account for the fact, for example, that an enzyme will hydrolyse glycyl-*dl*-alanine but not glycyl-*l*-tyrosine (Abderhalden and Brahm, 1908).

The study of the bacterial proteases had its origin in the controversies concerning putrefaction and sepsis centring around the names of Gaspard (1822), Panum (1856), Schwann (1837), Pasteur (1863), Bienstock (1899), Metchnikoff (1893), and many others. Preliminary observations on the intracellular digestion of Protozoa seem to have been made much earlier (Corti, 1774; Goeze, 1777)¹. Since that period a scattered and voluminous literature has accumulated. Whilst it has been possible to group the animal proteases broadly into peptases, tryptases

¹ An account of the above controversies in their historical setting may be found in the *System of Bacteriology*, 1, Medical Research Council, H.M. Stationery Office, London, 1930.

and ereptases, or more strictly the proteinases into pepsin, trypsin and cathepsin, with a group of peptidases included under the general term "erepsin," each with their particular zone of activity regarding pH and chiefly splitting a definite substrate, no such classification has as yet been possible for the enzymes of micro-organisms. The experimental difficulties of study are such that in very few cases has an exhaustive examination been carried out. The enzymes in question, although perhaps not less active, are more difficult to obtain in quantity and in a separate condition than the animal proteases. In the highly differentiated animal organism the production of a particular enzyme is largely localised, *e.g.* pepsin in the gastric mucosa and trypsin in the pancreas, and the enzyme can be separated by extracting the appropriate tissue. But in the lower organisms a complex mixture is produced by the single undifferentiated cell. With bacteria, the enzyme may be endocellular, it may be secreted into the surrounding medium, or an organism may possess both types which are not identical in properties. Since some bacteria so readily autolyse, doubt often arises as to whether the technique employed has been adequate to remove all the cells if the exo-enzyme was studied, or to make sure that the endo-enzyme was not liberated by autolysis of the cell protoplasm.

On the other hand, microbiology should have a unique contribution to make to the study of enzymes because the organism can in many cases be grown in a "synthetic" medium consisting of a few carefully purified substances of known composition. Such a medium must contain a source of nitrogen (ammonium chloride, ammonium phosphate, or an amino acid), a carbon compound readily utilisable as a source of energy (dextrose, glycerol, lactate), phosphates, and certain other ions as Ca^{++} , Mg^{++} , Cl^- , etc., the exact requirements probably varying with different organisms. In no other living system can the environment be so easily and exactly controlled and correlated with its effects on the production of enzymes. It is perhaps surprising that more attention has not been paid in the past to this point, but the explanation probably lies in the fact that bacteriology has been for so long the handmaid of pathology, and the more virulent pathogens are much more difficult to grow in simple media than the commoner saprophytes. Robinson and Rettger (1918), however, found that most organisms of a large number tested, including *Pneumococcus*, *Gonococcus*, *Meningococcus*, *M. catarrhalis*, and *B. pertussis*, could be grown in the protein-free digestion product "opsine," except the diphtheria group. Burrows (1933) this year reports the growth of the anaerobe *Clostridium botulinum* in a synthetic medium¹.

Moreover, it has been maintained by many workers that enzymes are only produced when the organisms are growing in complex media containing peptone and other substances of unknown composition. The literature on this point is mostly contradictory and it will be discussed in detail later.

In attempting to form a critical appraisal of the older investigations on bacterial enzymes it must be remembered that although Sørensen in 1909 indicated the dependence of enzyme activity on pH, it was not until 1917 that Clark and Lubs brought out their colorimetric method for the determination of pH, now widely

¹ For a full discussion of the requirements of bacteria for growth see Peckett, 1933.

used in bacteriology, and without control of pH adequate analysis of enzyme systems is impossible. Moreover, it is only within the last few years that an adequate technique has been worked out, chiefly by Willstätter, for separating the component parts of enzyme mixtures, so that, according to Haldane (1930), all properties ascribed before 1929 even to the better defined enzymes trypsin and erepsin are due to a mixture. In general, in reviewing any work on the subject the following would seem to be criteria of prime importance:

(1) Distinction must be drawn between effects which are due to actual multiplication of the organisms on the substrate, to the secretion of soluble enzymes into the medium, and to the liberation of endocellular enzymes by autolysis. In much of the very early work decomposition was probably a combination of all three.

(2) Due regard must be paid to the growth of the organisms before it can be said that they are incapable of attacking a given substrate. For example, in the early controversies concerning putrefaction (p. 239) bacteria were sown into flasks of meat and egg-white and similar substances. Exhaustive chemical analyses were carried out to follow the decomposition, if any, but in very few cases were any counts made to show that the bacteria had not simply died out under the conditions chosen.

(3) The concept of pH , its application to bacteriology and its fundamental importance in enzymic activity should be borne in mind.

(4) The importance wherever possible of careful purification of substances used in the medium should be remembered. The quantities necessary completely to change the metabolism of bacteria are exceedingly small.

(5) The significance of vitamins in nutrition must not be overlooked: discordant results with synthetic media may be due to their presence in amino acids or other compounds used.

(6) When using synthetic media it is necessary to wash the organisms and to take through a sufficient number of subcultures to ensure that spurious results are not being obtained by carrying over small amounts of impurities from the original culture.

(7) Before any final pronouncement can be made concerning the nature of bacterial enzymes they must be separated and purified and their characteristics, such as pH optima, substrates attacked, etc., determined.

II. THE BREAKDOWN OF NATIVE PROTEINS.

(1) *Putrefaction.*

Following on the observations cited above that bacteria are invariably present in putrid, evil-smelling fluids, and in abscesses and other suppurative conditions, it was generally stated that most bacteria could give rise to what was termed "putrefaction," and that the products of putrefaction—the so-called "ptomaines"—were responsible for the toxic effects of spoiled foodstuffs. Both these views have undergone considerable modification. Evidence for the toxicity of ptomaines was based on inoculation experiments, and Savage (1921) has shown that when putrid food is fed by the mouth it has few if any ill-effects. Food-poisoning is, in fact, the work of a few bacteria manufacturing specific toxins (Savage, 1920) and food apparently sound may contain a lethal dose while putrid food may be quite harmless. Also it has been found that far fewer organisms than was originally supposed are able to initiate changes in the protein molecule. Pasteur in 1863 was led to postulate

his famous dictum that putrefaction only occurred in the absence of free oxygen. He says (1863): "La conséquence la plus générale de mes expériences est fort simple, c'est que la putréfaction est déterminée par des ferments organisés du genre *Vibron*.... Il résulte de ce qui précède que le contact de l'air n'est aucunement nécessaire au développement de la putréfaction. Bien au contraire, si l'oxygène dissout dans un liquide putrescible n'était pas tout d'abord soustrait par l'action d'êtres spéciaux, la putréfaction n'aurait pas lieu." Emmerling, however (1897), claimed to have brought about extensive putrefaction of egg-albumin and blood fibrin with the aerobes, *Staphylococcus pyogenes* and *Streptococcus pyogenes*, putrefaction of wheat gluten with *Proteus vulgaris*, and gelatine and blood fibrin with *B. fluorescens* (Emmerling and Reiser, 1902). Brieger (*Ueber Ptomaine* (1885), quoted by Emmerling), on the contrary, stated that albumins were attacked with the greatest difficulty by pus cocci. Detailed investigations were made by Nencki (1889) and Bienstock (1901). The latter sowed some twenty facultative aerobes into sterile blood fibrin, and he agreed with Pasteur that the aerobic bacteria do not bring about putrefaction of fibrin. "That is the specific work of obligate anaerobes. Without them putrefaction does not take place. The aerobic organisms are the natural assistants of the anaerobic putrifiers. They make their existence possible, perhaps by the removal of oxygen or other means, and partly by converting the fibrin into a soluble form available for further decomposition. The whole group of indol-forming bacteria may belong to this group of aerobes" (1899). Bienstock found (1901) that almost everywhere—in garden soil, street dirt, scrapings from old harness—he could isolate an anaerobe called by him *B. putrificus*¹, and he postulated that true putrefaction was always due to this or similar obligate anaerobes. It is to him that we owe the definition of putrefaction as now generally understood, *i.e.* the bacterial decomposition of protein matter to form foul-smelling substances, "Fäulnisprodukte." Simple cleavage of the protein molecule of the nature of hydrolysis may be accomplished by the enzymes of a larger number of organisms including some aerobes, but is to be distinguished from true putrefaction (see, however, p. 258).

Tissier and Martelly (1902) followed the changes taking place in butchers' meat when bought freshly from the shop and allowed to incubate at 20° C., without any further addition of bacteria. They state that meat so bought contains all the organisms necessary for its complete putrefaction, many of which will, however, only multiply when conditions become favourable. During the first few hours, with the meat stored in air, fermentation of the sugars present takes place with growth of the aerobes, which assist in setting up anaerobic conditions for the later multiplication of the anaerobes. Little breakdown of the albumin occurred in this period. After 24 hours proteoses, leucine, tyrosine, amines and ammonia could be detected. The mixed flora present contained *Micrococcus flavus* liq., *Staphylococcus albus*, *B. coli*, *Streptococcus pyogenes*, *Diplococcus griseus* and *B. filiformis*. After 3-4 days the acids formed are neutralised by the production of ammonia,

¹ Later workers, using improved technique, have stated that this was in fact a mixture of several anaerobes.

and protein breakdown is more marked. The odour becomes definitely putrid and the strict anaerobes *B. perfringens*, *B. bifementans sporogenes* can now be found in numbers. In 8–10 days all the sugars have disappeared, the saponified fatty substances have become ammonium soaps, the glycerol liberated is oxidised and the odour is foetid. Phenols, indol, hydrogen sulphide, amines and ammonia are liberated, and *B. putridus gracilis*, *B. putrificus*, *Diplococcus magnus anaerobicus* and *Proteus Zenkeri* multiply vigorously. Finally the anaerobes either sporulate or disappear, the aerobes become less numerous, and a viscous black mass is left containing insoluble residues but with all the available protein and peptone broken down.

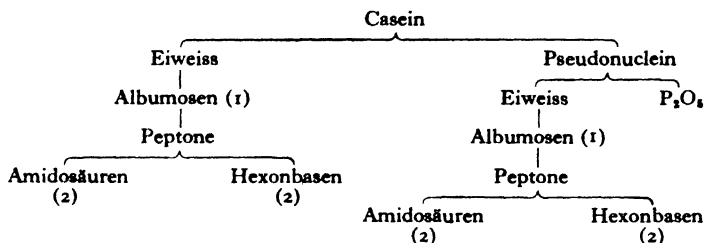
Rettger (1906) made up flasks containing sterile chopped beef and coagulated egg-white neutralised with sodium carbonate. Into these he sowed separately *B. coli communis*, *B. lactis aerogenes*, *B. fekalis alkaligenes*, *Proteus vulgaris*, *B. pyocyaneus*, *B. fluorescens*, *B. cloacae*, *B. prodigiosus*, *Staphylococcus pyogenes*, *Micrococcus cereus*, *B. putrificus*, the bacillus of malignant oedema and the bacillus of symptomatic anthrax. Anaerobic conditions were set up by passing in hydrogen, and after due incubation analyses were carried out for various decomposition products as indol, skatol, phenols, various oxy and amino acids, hydrogen sulphide and mercaptan. It was concluded that only the three obligate anaerobes decomposed the egg-meat mixture, which they did rapidly and thoroughly. It is not, however, stated whether any counts were made to follow the rate of multiplication of the bacteria, so that many of them may have died out under these conditions. This criticism applies in general to much of the work of about this period. Further experiments along similar lines were carried out later by Rettger (1908). He specifically states that true putrefaction is the work of obligate anaerobes, but not all anaerobes cause putrefaction, *e.g.* *Clostridium tetani* has little action in this respect. Decomposition of protein by ordinary digestion is however recognised. "No one can deny that much of the decomposition of albuminous matter is carried out by the obligate aerobes (*B. subtilis*, *B. mycoides*, etc.), but such transformation is one of ordinary dissolution or digestion. Free oxygen is always needed in abundance....Decomposition of albumin by the aerobes is never accompanied by the foul odours which are so characteristic of putrefaction."

(2) *Aerobic decomposition.*

Martin (1890) seems to have been one of the first to abandon the meat stews beloved of the older bacteriologists in favour of purified substances. He sowed anthrax bacilli into a solution of pure alkali albumin together with "mineral salts of the composition of the salts of serum," and after 10–15 days' incubation filtered the culture through a Chamberland candle. In the filtrate he found albumoses, peptone, "an alkaloid," and leucin and tyrosin. Exact details of the methods of purification and of the composition of his media were not however given.

Taylor (1902) prepared pure casein solutions in bulk, the casein having been dried and sterilised in the presence of chloroform vapour before dissolving. Growths of *B. coli* and *Proteus vulgaris* were scraped off solid media so as to carry away as

little nutrient as possible and inoculated in batches of the casein solution. Analyses for the various split products were carried out using Fischer's methods. It was concluded that casein was broken down according to the following scheme, *B. coli* only carrying the process to (1) and *B. proteus* right down to (2):



An important step forward was taken by Bainbridge (1911). To the following medium:

Sodium chloride 0.5 per cent.,
 Sodium sulphate approximately 0.1-0.25 per cent.,
 Calcium chloride trace
 Potassium phosphate trace } approximately 0.1 per cent.,

he added 0.1-0.5 per cent. of purified crystallised egg-albumin, serum albumin or globulin, or alkali albumin. After having been made slightly alkaline to litmus, the medium was inoculated with *B. coli communis*, *B. enteritidis*, *B. typhosus*, *B. proteus*, *Staphylococcus pyogenes aureus* and *Gonococcus*. Counts were carried out and the protein estimated by coagulation and weighing, the periods of incubation varying but often from 8 to 24 days. Bainbridge found that, provided the protein was free from salts of ammonium, little or no multiplication of the bacteria took place. The organisms were unable, in the absence of a source of energy or of a simpler source of nitrogen, to bring about breakdown of the protein. If a little peptone were added vigorous multiplication took place, but even then no decomposition of the protein occurred except with the *Proteus*. Two points should be noted in connection with this work. Firstly, salts of magnesium were absent and there is reason to believe that they play an important rôle in the growth of bacteria in simple media (p. 255). Secondly, the periods of incubation were such that considerable autolysis could have taken place, for according to Rettger (1905) this may occur in from 2 to 3 days. Thus none of the organisms tested, except *Proteus*, are able to synthesise an enzyme attacking native proteins, even when growing vigorously. There remains, however, the possibility that some specific ion or other substance (e.g. some particular amino acid) essential for enzyme production, was absent from the medium. Sperry and Rettger (1915) confirmed this point of view. They used serum albumin and edestin in addition to egg-albumin, all carefully purified. There was little bacterial development in any medium made up with pure protein and a few salts (salts of magnesium however again absent), using *B. subtilis*, *B. anthracis*, *B. pyocyaneus*, *B. prodigiosus*, *Proteus vulgaris* and *mirabilis*, *B. coli*, *B. typhi*, *B. pullorum*, *Staphylococcus pyogenes aureus*, and in addition the

obligate anaerobes *B. putrificus*, *B. anthracis symptomatici* and *B. oedemati maligni*. The following conclusions were reached: "The inability of bacteria to decompose native proteins is not limited to aerobes and facultative anaerobes, but even well-known and extremely active putrefactive anaerobes are devoid of this property. Solutions of native proteins may undergo complete proteolysis, however, if they contain peptone or some other nitrogenous food material which readily furnishes the necessary nitrogen for bacterial development. In such instances the proteolysis of the native protein is the immediate result of the action of an enzyme which has been elaborated by the bacteria during the process of rapid multiplication. This multiplication is made possible by the nitrogen containing material which is present along with the native protein.

"The resistance of native proteins to direct decomposition by bacteria is not due to any antiseptic properties of the proteins, but to a constitution of the molecule which renders it relatively stable, the component parts being so firmly bound together that a strong cleavage-producing agent, such as extreme heat, strong acids and alkalis, and enzymes, is required to change them so that bacteria may utilise their products for cell nutrition."

Rettger, Berman and Sturges (1916) carried the matter a stage further when they showed that heat-coagulated albumin, albumoses, and peptones are not available for direct utilisation until further decomposed by enzyme action. If a simpler source of food material were added, the proteose was attacked by *B. subtilis*, *B. ramosus*, *B. proteus* and *B. prodigiosus*, but not by *Staphylococcus aureus* or *albus*, or by the *coli-typhosus* group.

In connection with the above statements of Sperry and Rettger it is perhaps not out of place to observe that fresh egg-albumin apparently does possess some germicidal power apart from its resistance to breakdown, due to a toxic diffusible substance (Sharp and Whittaker, 1927) which may possibly be identical with Fleming's lysozyme (Fleming, 1922). There is also an earlier observation of Hankin (1890) that Halliburton's cell globulin- β extracted from lymph glands possesses the power of killing bacteria.

The foregoing is an outline of the study of the gross effects of bacteria when in contact with proteinaceous material. Having established the fact that proteins are not in general directly utilisable by bacteria until they are broken down by their enzymes into simpler substances, the problem now resolves itself into a study of the conditions under which the micro-organism can marshal its enzymes for this attack. This parallel line of investigation was begun about the last decade of the nineteenth century, and will now be considered in some detail.

III. BACTERIAL ENZYMES.

(1) *Soluble enzymes found in the medium.*

Recognition of the fact that the changes induced by micro-organisms are brought about by the enzymes which they elaborate emerges chiefly from the writings of Pasteur, Liebig and Buchner, circa 1860-1900 (see Stephenson, 1930, p. 3). These

workers were mostly concerned with sugar fermentations. An early observation suggesting that protein breakdown was also due to enzyme action is that of Schutzenberger (1874), who found that beer yeast maintained for 12–15 hours at 35–40° C. lost 8–9 per cent. of its solid matter, which went into solution, and he postulated that the yeast had “a physiological action on its own substance.” Bitter (1886) made an important advance when he showed that liquefaction of gelatine and serum proteins could be brought about by the fluid in which the bacteria had been grown, the presence of the living cell not being necessary. “*Vibrio Koch*” and “*Vibrio Proteus*” were used. The former is very sensitive to heat, half an hour at 60° C. rendering it incapable of further multiplication. Cultures made in flasks containing meat broth so heated and tested for sterility still possessed the property of liquefying gelatin. Coagulated proteins were also attacked by the sterile cultures and the presence of a diastatic enzyme was noted. He found that best liquefaction occurred in alkaline solution, some in neutral solution and only a little in acid solution.

This point of view was further emphasised by Brunton and Macfadyen (1889). They grew Koch's comma spirillum, Finkler's comma spirillum, a putrefactive micrococcus, Klein's scurf bacillus and Klein's Welford bacillus in meat broth for periods of some days. Heating to 60–70° C. killed the bacteria, but the fluid still possessed the property of liquefying gelatin. Moreover, a precipitate could be formed by adding alcohol which on solution in chloroform water liquefied gelatin and fibrin. Fermi (1890, 1891) studied the enzymes attacking gelatin and fibrin from a number of bacteria, and made observations on the temperatures inactivating the enzymes, the effects of adding acid and alkali and various salts. Egg-albumin and blood serum were found to be resistant to attack. He could not obtain the enzymes when the bacteria were grown in protein-free sugar-salt solutions. He also showed that the presence of living cells was not necessary for the liquefaction of gelatin, since cultures heated sufficiently to kill the organism would still liquefy. In later work however (1892) *Micrococcus prodigiosus* and *B. pyocyaneus* yielded a liquefying enzyme when grown in a medium containing ammonium and potassium phosphates, magnesium sulphate and glycerin or cane sugar.

Fermi made experiments with a variety of simple media in an endeavour to answer the question “Auf welchen Nährboden bilden die Mikroorganismen ihre proteolytischen Fermente und auf welchen nicht?” As, however, with many of the sugars employed large amounts of acid were probably developed, which apparently were not neutralised before testing for the enzyme, his results must be viewed with caution.

Macfadyen (1892) and Hahn (1898) showed that enzymes could be extracted from the bacterial or yeast cell apart from those which could be found in the cell-free medium. The former extracted the cells of Koch's comma bacillus, Denebe's cheese spirillum, *Vibrio Metchnikoff*, and Finkler and Prior's spirillum grown on agar with chloroform water or 40 per cent. glycerol, which completely dissolved them in 3 or 4 days at 30–39° C. The extract, rendered sterile by the addition of thymol or menthol, liquefied gelatin and digested fibrin. Egg-albumin

was also disintegrated in 2-5 days at 39° C. Macfadyen concluded that bacteria which liquefy gelatin do so by means of a soluble ferment acting apart from the cells, and that the amount of the enzyme secreted varies according to the soil, meat-broth cultures giving the greatest activity. The action of the ferment also varies according to the soil, or substrate in modern terminology, the cholera enzyme having a more energetic action on egg-albumin than on fibrin, and Finkler's spirillum having the opposite effect. No definite distinction was however drawn between the nature of the enzyme obtained from the cells and that found in the medium. Hankin and Wesbrook (1892) controverted Fermi's statement that the anthrax bacillus did not produce a proteolytic enzyme. They state that his method of immersing pieces of fibrin in a tube was not sufficiently sensitive, and they were able to show that an enzyme was secreted which liquefied gelatin and broke down proteins to albumoses. The albumose obtained from the organism by enzyme action was not the same as the "toxalbumin" which could confer immunity to anthrax.

Emmerich and Löw (1899) worked on bacterial enzymes as a cause of acquired immunity. They showed that old broth cultures contained enzyme-like bodies tending to dissolve bacteria. *B. pyocyaneus* was studied chiefly, and the substance obtained from old filtered and concentrated broth cultures was called "pyocyanase." It had the property of dissolving anthrax bacilli *in vitro*. Much subsequent work has been done on the immunological question here raised, but it is outside the scope of this paper.

Schröder (1902) allowed yeast to autolyse in presence of ether. He found that the filtered solution after autolysis contained a soluble protein which could be precipitated and which resembled albumin. Rettger (1905) also studied autolysis.

Eijkman (1901, 1904) planted colonies of bacteria on plates containing emulsified milk or various proteins and could distinguish a diffusible enzyme by a clear zone round the colony. He postulated a casein-splitting, a haemolytic, an amylolytic and a lipolytic enzyme in many of the organisms. Among the organisms giving a clear zone of milk agar were *B. anthracis*, *B. pyocyaneus*, *Staphylococcus pyogenes*, *Vibrio Metchnikovi*, *V. cholerae*, *B. fluorescens*, *B. prodigiosus*, *B. subtilis*, *B. megatherium*, and *B. mesentericus*. Among those not digesting the casein were *B. typhi*, *B. coli communis*, *B. mallei*, *B. pestis*, *B. diphtheriae*, and *B. lactis cyanogenes*.

Hata (1904) grew *B. prodigiosus* and *B. fluorescens* in sterilised milk, and separated the enzymes by precipitation with alcohol and ammonium sulphide (Schwefel-ammonium). He found that the activity of the enzyme was lost with repeated purification, but by precipitating two or three times he was able to obtain a preparation which readily liquefied thymol-gelatin on incubation at 35° C. and which also coagulated the caseinogen from milk containing 1 per cent. phenol. *B. prodigiosus* gave the more active preparation, its trypsin-like enzyme attacking gelatin being about three times as active as that from *B. fluorescens*, and its rennet-like enzyme about five times as active. Nicolle (1907) using *B. subtilis* showed that when grown in their presence it liquefied gelatin, coagulated serum and egg-albumin, and that it had a "clearing" action on milk. Filtrates from peptone broth cultures were, however, inactive on the serum, albumin and milk, but liquefied gelatin and

haemolysed rabbit's red blood corpuscles. Filtrates also had the property of dissolving other organisms. Nicolle postulated a trypsin and a gelatinase elaborated by this bacterium, the former being associated with the cell and the latter liberated into the medium. De Waele (1909) attempted to digest various bacteria with trypsin, and he postulated that together with the proteolytic activity of bacteria developed chiefly in the medium, there was also an anti-proteolytic element associated more closely with the cells, thermolabile above 65° C. but rather more thermostable than the protease.

Mesernitzky (1910) followed out the decomposition of gelatine by *Micrococcus prodigiosus*. He used broth cultures filtered through a Chamberland candle, and he followed out the process of liquefaction by chemical means. It was concluded that secondary products are first formed, precipitable with tannin, then simple peptides and a crystalline product identical with glycine. Von Gröer (1912) carried out extensive studies on the gelatinase obtained from cultures of *prodigiosus* by following changes in the viscosity of centrifugates from broth cultures together with gelatine. The gelatinase was found to be sensitive to acids and sodium fluoride, and fairly resistant to heat, gelatine however exerting a protective effect on the enzyme against the destructive effect of sodium fluoride or heat. Sasaki (1912) found that *B. coli* was able to split the dipeptides glycyl-tyrosine and glycyl-glycine, as also were other members of the colon-typhoid-dysentery group, some gelatin liquefiers and *Micrococcus tetragenus*.

Bertiau (1914) also developed a semi-quantitative method of estimating the liquefaction of gelatin, by mixing measured amounts of the enzyme solution, obtained either by filtration or by precipitation from the original culture with alcohol and re-solution in physiological saline, with molten gelatin and incubating at 37° C. *B. prodigiosus* and *B. subtilis* were chiefly studied. Moreover, by immunising rabbits an active anti-ferment was obtained which specifically and completely neutralised the gelatinase. He concluded that bacterial gelatinase is not to be identified with trypsin, as the anti-ferment experiments show.

Corper and Sweany (1918) added toluene or chloroform to broth cultures of tubercle bacilli and found that there was present a trypsin-like enzyme which split proteins in alkaline solution, an erepsin-like enzyme capable of decomposing peptone in acid solution, a weak pepsin-like enzyme splitting proteins in acid solution, a nuclease and a urease. Dernby (1917) after concentration of yeast autolysates postulated the presence of three enzymes, yeast pepsin, yeast tryptase, and yeast ereptase with pH optima of 4·4·5, 7·0 and 7·8 respectively. He further studied filtrates from the tubercle bacillus, pneumococci, streptococci, certain staphylococci and the tetanus bacillus, all of which gave no proteolysis. Filtrates from cultures of *B. subtilis*, *B. pyocyaneus*, *B. proteus*, *B. prodigiosus*, *B. sporogenes* and *B. histolyticus* showed strongly active proteolysis. It was not however possible to group the enzymes of these organisms in the same manner as the yeast proteases. Activity was present within the range pH 4-9, the optimum being pH 6-7.

The French workers Blanc and Pozerski (1920) have contributed interesting studies on the enzymes of the strict anaerobes *B. sporogenes* and *B. histolyticus*.

They started from the observation of Weinberg and Séguin that the shock following gas gangrene has been attributed to resorption by the organism of the products of digestion taking place at the site of the wound. A broth made from putrid meat of pH 7 was found to be the best medium for growing these bacteria, together with added calcium sulphide. The proteolytic action of the whole culture was studied after saturation with chloroform for 10 minutes, and also of filtrates through a Chamberland candle. Cultures and filtrates rapidly liquefied gelatin, the optimum pH being 5.5, *B. histolyticus* being however more active than *B. sporogenes*. Digestion of coagulated ovalbumin and serum proteins took place with the cultures, but very slowly with the filtrates. A rennet-like enzyme was demonstrated which coagulated fresh milk, but only coagulated heated milk when calcium salts were added. Filtrates contained an active casease which was inhibited by the addition of salts of calcium. Cultures digested native ovalbumin and serum albumin but the filtrates were without action on these proteins. Differences were observed in the behaviour of the two organisms. Neither cultures nor filtrates of *B. sporogenes* had any action on the fresh muscle of the guinea-pig. Doses of this organism injected into a guinea-pig only provoked fleeting oedema without digestion of the muscle. But if that injection were preceded by several drops of lactic acid, a big abscess appeared leading to digestion of the tissues around it. *B. histolyticus* on the other hand did digest fresh muscle, broth cultures and filtrates, and injection of this bacterium alone led to all the symptoms described by Weinberg and Séguin, without previous denaturation of the protein by injection of lactic acid. The whole cultures formed amino acids in their digestion products, whilst filtrates only decomposed substrates as far as peptones. These authors state that the enzymes of the two bacteria must be different since anti-sera prepared from horses were specific for each. Their pH optima were 5.5 as contrasted with 1.8 for pepsin, and they were precipitated by safranin in a similar manner to trypsin.

Further work on the anaerobes was carried out by Dernby and Blanc (1921) who found that filtrates from the following organisms grown in veal broth with calcium sulphide displayed strong proteolytic activity for several months. The organisms were *Clostridium sporogenes*, *C. histolyticum*, *C. canadiense*, *C. putrificum* and *C. perfringens*. Growth took place from pH 5 to 9, with its optimum at pH 7.0, while gelatin was liquefied and peptone digested by the filtrate at pH 4-8, optimum at pH 6. It was concluded that a tryptase was present. Kendall and Keith (1926) working with *B. proteus* obtained a soluble bacteria-free enzyme rapidly digesting gelatin, more active than commercial trypsin but resembling it in effect. Schierge (1926) stated that a protease obtained from *B. coli* and also a putrefactive organism split casein more easily than peptone, and gelatin less easily. The coli protease resembled trypsin except for its pH optimum 6-6.6. The proteins of both active and inactivated sheep serum were resistant to the protease, and there was no relation between the growth of the organism and its ability to split peptone. The same author later (1928) sowed *B. coli* into solutions containing casein, and from the decomposition products he isolated hexone bases and monoaminodicarboxylic acids, concluding that this organism produces a protein-splitting enzyme having optimum

action at pH 6-7.5. This statement conflicts with that of Berman and Rettger who maintained that the colon group did not possess enzymes capable of attacking any higher combination than peptones. Gross (1928) working with a *Staphylococcus* could obtain an enzyme in filtrates from the cultures which coagulated citrated blood, but he was unable to demonstrate the presence of a proteolytic enzyme.

(2) *Endocellular enzymes.*

Otsuka (1916) devised a method of attempting to differentiate between the action of the soluble enzyme and that contained within the cell. He first of all showed that *Staphylococcus pyogenes aureus* and *B. prodigiosus* could decompose gelatin, blood serum and dipeptides when grown in their presence. But when the cells were removed by filtration through a Chamberland candle the filtrate was no longer active towards dipeptides but still attacked the proteins. If the cells were shaken with toluene so that they no longer were capable of growth, they still attacked both proteins and dipeptides. Evidently the enzyme responsible for rupture of the dipeptide linkage is associated with the cell and is not liberated into the medium. Berman and Rettger (1916) further studied this erepsin-like enzyme. They found that *B. coli* had little if any action on peptone over short periods but that with further incubation slight amounts were destroyed. They showed that this was due to breakdown of the polypeptide fraction of the peptone and not the proteose fraction. The enzyme was similar to animal erepsin except that it did not attack casein. Stevens and West (1922, *a, b*) disintegrated the cells of a haemolytic *Streptococcus* suspended in a phosphate buffer by grinding in an agate mortar. Sterility was ensured by the presence of toluene and the preparation centrifuged at the end. An enzyme preparation was obtained active between pH 4.4 and 8.7 with its optimum at 7.2, attacking peptone and casein but not serum albumin. Avery and Cullen (1923) lysed suspensions of pneumococci at 0° C. with bile, to which this organism is very sensitive. Peptone was chiefly used as substrate as it was readily attacked. Casein and fibrin were also broken down but albumin and gelatin were not attacked. The optimum was at pH 7 and heating for 10 min. at 100° C. destroyed the activity. The velocity of hydrolysis is stated to be proportional to the enzyme concentration, and weight for weight this "peptonase" was estimated to be several times as active as the usual commercial dried enzyme preparations. Dernby and Siwa (1922) found that when diphtheria bacilli are grown in broth, proteins and peptone are split. This splitting does not begin immediately after growth but lags behind some 4-6 days, when the organisms are apparently autolysing. Little or no enzyme can be detected in filtered broth from young cultures, but a weakly active enzyme was found in macerated autolysed bacilli.

Neil and Fleming (1927) extracted the enzymes of *Meningococcus* by freezing and thawing and filtration through a Berkefeld candle. Peptone was hydrolysed to peptides and amino nitrogen, and since other proteolytic properties could not be found they concluded a "peptonase" was present, which was thermolabile.

Glinka-Tschernorutzky (1929) studied the enzymes of *B. mycoides*. Little or no action could be obtained from filtrates of the cultures in peptone water, but

enzymes were extracted, from the dried bacteria, after treatment with acetone and ether by grinding with quartz sand. A number of proteins were used as substrates, but peptone and gelatine were most attacked. It was concluded that a tryptase was present, and a "protein-sparing" action was noted when glucose was added to the medium.

Tarnanen (1930) found that killed suspensions of *Bact. casei* rapidly digested casein, gelatin and peptone. The optimum pH was 6 and temperature 42° C. Pre-treatment of the culture to a pH < 4.5 slowly inactivated the enzyme. Autolysis with 50 per cent. glycerol extracted the enzyme. Chloroform was found to inactivate the enzyme and phosphate inhibited its action on casein and gelatin but not on peptone. Alumina adsorption gave rough separation of a protease and a polypeptidase, the latter splitting both peptones and dipeptides, optimum pH 6.8. The activity of the protease was slightly increased by hydrocyanic acid.

Virtanen and Tarnanen (1931) later carried out a study of the enzymes of *B. fluorescens liquefaciens*. They were able to separate from filtrates of cultures of this organism a proteinase cleaving casein and gelatin without the formation of amino groups. From the dried bacteria they extracted enzymes splitting peptone and dipeptides respectively, these enzymes being only formed with difficulty from cultures autolysed in the presence of toluene. The same authors (1932) made further observations chiefly with *Pseudomonas fluorescens* and *B. subtilis*. They found that the former of these secretes a proteinase into the medium while it is still actively multiplying. They used the word "secrete" advisedly since they concluded that little or no autolysis had occurred, and moreover, a polypeptidase and a dipeptidase was obtained from the bacterial residue after centrifuging which was not detectable in the medium until after considerable autolysis had occurred. The proteinase was somewhat thermostable if heated in the presence of protein; it withstood several minutes' heating at 100° C. This method of alumina adsorption developed by Willstätter has led to valuable results in the case of yeast which will now be discussed.

IV. THE ENZYMES OF YEAST.

Early work on this organism, apart from examples cited previously, was carried out by Will (1898), Geret and Hahn (1900), Will (1901), Bokorny (1902) and Vines (1904). Geret and Hahn used pressings from fresh yeast and then estimated its power of digesting the coagulum from yeast and fibrin. They concluded that the preparation was most active in slightly acid solution, that its activity was increased by the addition of neutral salts and slightly reduced by such antiseptics as chloroform, toluene, etc. Vines stated that two enzymes were present in yeast, one readily soluble in water which did not attack fibrin but effected peptolysis rapidly, the other soluble in 2 per cent. sodium chloride and readily attacking fibrin.

The work of Dernby, already quoted, suggested the presence of three proteolytic enzymes in yeast autolysates; these experiments were made however before the development of Willstätter's adsorption technique, which has yielded clear cut results in this, one of the few cases in which careful purification and separation of

the enzymes of micro-organisms has been carried out. The method consists in adsorbing the enzymes from concentrated autolysates on aluminium hydroxide gels from which they can be eluted with phosphate solutions at a critical pH and fractional separation and purification carried out. Willstätter and Grassman (1926) state that yeast contains three proteolytic enzymes, one acting on proteins and peptones, one on polypeptides, and one on dipeptides. The first has its pH optimum at 5.0, the latter at 7.0–7.8. Grassman (1927) found that all the proteins investigated—egg-albumin (native and denatured), gelatin, casein, albumin, peptone—were hydrolysed at pH 5.0 by yeast trypsin except native egg-albumin. Hydrolysis was measured in terms of cubic centimetres of alcoholic potash required to titrate back to a given pH. This resistance of native proteins to attack which has been so frequently noted is therefore due to the inability of the enzyme to initiate breakdown of the intact protein molecule. Some change in the structure of the molecule on denaturation must take place making it more accessible to the enzyme, since the denatured protein is attacked. Grassman and Dyckerhoff (1928) propose a revision of nomenclature on the subject. The word “protease” has been used to connote so many different enzymes that they propose to use the terms *proteinase* for enzymes responsible for the first rupture of the protein molecule, and *peptidase* for enzymes acting on a substrate which is known to be a peptide. These workers separated a tryptic and ereptic component, or a proteinase and a polypeptidase respectively, from pressed yeast. The tryptic and ereptic components were superficially similar to animal enzymes, but there were marked differences in detail. The action of yeast erepsin is limited to the hydrolysis of dipeptides so that it is really a dipeptidase, whilst animal erepsin may attack tripeptides or higher. Furthermore the yeast trypsin obtained after adsorption is really a mixture of a proteinase and a polypeptidase. Using this nomenclature, according to their results yeast contains one proteinase, a polypeptidase, and a dipeptidase, and not two proteinases as Dornbush postulated. The proteinase is liberated early in the growth of the cell, in between 15 and 48 hours, whereas the polypeptidase is slowly liberated by autolysis in the presence of chloroform in slightly alkaline solution, and is not complete even after a week. Willstätter controverts the view of Waldschmidt-Leitz that the specificity of these peptidases depends on the length of the polypeptide chain of the substrate. He maintains that length of the chain as such is not the controlling factor, but the acidic and basic properties of the free —COOH and —NH_2 groups, which are nearest together in a dipeptide.

V. THE ENZYMES OF FUNGI AND PROTOZOA.

With regard to the enzymes of fungi, there are hints in the literature suggesting that their mode of action is rather different from that of bacteria. The point does not seem to have been followed up. Bainbridge (1911), for instance, records that he was led to his work on the bacterial breakdown of proteins from the observation of Martin that when flasks of pure albumin were left about the laboratory no bacterial growth occurred but often there was good growth of contaminating fungi.

If further investigation should prove the correctness of this view, it would seem that fungi are able to initiate breakdown of native proteins in a way fundamentally different from bacteria.

Hansen (1889) showed that *Penicillium glaucum* brought about liquefaction of 5-7 per cent. gelatin gels when grown upon them, and found that a glycerol extract of the fungus also liquefied gelatin, particularly in neutral solution. Bourquelot (1893, a, b, 1894, 1897) in a series of studies grew *Aspergillus niger* on Raulins' medium, which contains cane sugar, tartaric acid, ammonium nitrate, ammonium phosphate, potassium carbonate, magnesium, iron, zinc and ammonium sulphates and potassium silicate.

An extract was prepared by grinding with sand in the presence of chloroform water, which broke down fibrin and coagulated egg-white to the stage of peptones; the action of the enzyme was feeble in acid solution and hence it was concluded to be tryptic in nature. Malfitano (1900) also grew *A. niger* on Raulins' medium. He distinguished between "le proteolyse" which attacked true proteins, and "la diastase proteolytique" which liquefied gelatine, and he found that changing the composition of the medium did not affect the enzyme production so long as adequate growth was obtained, on which it primarily depended.

Butkewitsch (1900) cultivated *A. niger*, *Penicillium glaucum*, and species of *Mucor* on peptone or fibrin plus cane sugar, phosphate and mineral salts. Leucine and tyrosine were isolated, showing that those fungi could attack both peptone and fibrin. Chrzaszcz (1901) studied the enzymes of some *Mucors*, chiefly with regard to liquefaction of gelatine.

Mazé (1905) found that *Penicillium candidum* and *P. glaucum* rapidly broke down the caseinogen of milk. Abderhalden and Pringsheim (1909) cultivated *Allescheria Gayonii*, *Rhizopus tonkiniensis*, *Aspergillus Wentii* and *Mucor mucedo*. They extracted enzymes by pulverising with quartz sand, mixing with Kieselguhr and pressing hydraulically. Various substrates were used and it was shown that not only was the naturally occurring *d*-alanine attacked but also *l*-alanine, which is unknown in nature. Extracts from the first three fungi split *l*-leucyl-*d*-leucine, while extracts from *Mucor* did not. Considerable impetus was given to the study of the enzymes of fungi from the elucidation of the part they play in the ripening of cheese. Dox (1909) carried out extensive studies on *Penicillium camemberti*. He states: "At the outset it may be noted that the enzymatic characteristics of these lower organisms are modified to no inconsiderable extent by the character of the culture medium in which they develop....The changes in content of enzyme are, however, essentially quantitative in character. There is no evidence that enzymes not normally formed by the organism in demonstrable quantities can be developed by special methods of nutrition." An inorganic medium with sodium nitrate, cane sugar, potassium phosphate and chloride, ferrous and magnesium sulphates, was used in which to grow the organism for about 10 days. The mycelium was removed, washed in running water, hashed up, washed with large volumes of acetone twice and finally with ether, and then dried and powdered. This preparation retained its activity for a year or more. Dox believed the enzyme to be closely related to

erepsin. It did not attack native proteins such as fibrin and ovalbumin, but it did break down casein, gelatin and proteoses, having its maximum activity in neutral or faintly acid solutions. Reed and Stahl (1911) demonstrated the presence of an ereptic enzyme after the growth of the parasitic fungi *Glomerella rufomaculans* and *Sphaeropsis malorum* in Dunham's solution. Gelatin was liquefied and tryptophane obtained from peptone. Scales (1914) studied the enzymes of *Aspergillus terricola* from the viewpoint that this and other fungi break down organic matter in the soil which then serves as nutriment for bacteria and plants. Extracting the enzyme from the dried mycelium in a similar way to Dox, he found that coagulated egg-albumin was attacked, as were also peptone, gelatin and milk. Bobilioff-Preisser (1916) studied the enzymes of species of *Oospora*, and Dox (1913) and Dox and Maynard (1912) followed the autolysis of cultures of *Penicillia* and *Aspergilli*. Franceschelli (1915) grew *Penicillium glaucum* on Kahlbaum's pure starch with the addition of certain salts. He stated that proteolytic enzymes could be obtained by growth in protein and fat-free starch solutions, acting in neutral or slightly acid solution, dialysing very slowly, and digesting proteins as far as tryptophane and ammonia. Waksman (1918) grew a variety of fungi in Czapek's medium using either peptone or sodium nitrate as the source of nitrogen. He concluded that the enzymes formed differ from animal proteases in having a greater range of optimum activity and a lower temperature optimum. They were not precipitated by safranin as is animal trypsin, and they will pass through Pasteur-Chamberland candles. Enzymes were obtained on protein-free media but were not so active as those from protein solutions. Fibrin and crystalline egg-albumin were decomposed by both the exo- and endo-enzymes of the organisms tested, as were also casein and peptone. Waksman and Lomanitz (1925) used a synthetic medium containing potassium phosphate, magnesium and ferrous sulphates, and sodium chloride plus protein or amino acid and dextrose. *Trichoderma koningi*, *Zygorhynchus mölleri*, an *Actinomyces* and various bacteria were worked with. The fungi all attacked casein when it was the only source of nitrogen in the medium, and the various amino acids also served for sources of both nitrogen and carbon. Rosenthal (1925) showed that *Tyrothrix scaber* when sown into tubes of the cholera vibron completely digested it in 5-6 days.

With regard to the proteolytic enzymes of Protozoa, many observations on the ingestion of particles of food by *Amoeba* and other organisms were carried out in the last two decades of the nineteenth century. A summary of many of these may be found in a paper by Greenwood (1886). The classical work of Metchnikoff (1884) on phagocytosis belongs to this period. Concerning the enzymes proper, Krukenberg (1878) made extracts of the plasmodium of *Aethalium septicum*, a Myxomycete, and found that a pepsin-like substance was present, since it digested fibrin in the presence of lactic or hydrochloric acids, but not in neutral or alkaline solution. Greenwood (1886) concluded that with *Amoeba proteus* digestion is accomplished by a non-acid fluid secreted in response to the presence of nutritive material, and not in response to such particles as starch grains. She later (1887) states: "Nutritious bodies are, when taken in by either animal (*Amoeba* or *Actinospherium*),

digested by fluid which is poured round them. This fluid separates a prey from the substance of the animal which has effected its capture, although it may upon occasion lie within a cuticle or within a cellulose or siliceous cell-wall. Upon these investments it has no action. Starch and fat are apparently not dissolved by it. It is colourless, neutral, active upon coagulated but more active upon non-coagulated proteid-matter." Metchnikoff (1889) concluded that *Myxomycetes* secreted acid in order to form a suitable medium for the digestion of albuminous matter by a peptic enzyme. Dantec (1890, 1891) stated that various Protozoa carried out digestion in the vacuoles, and that an acid secretion took place no matter with what the vacuole was stimulated. Greenwood and Saunders (1894) however came to the conclusion that the outpouring of acid was unaccompanied by any change in nutritive matter, ingesta in fact being stored for several hours before being dissolved. Hartog and Dixon (1893) experimented with *Pelomyxa palustris*, a large protozoan which may attain 2-3 mm. in diameter. The organisms were collected and treated with 95 per cent. spirit, dried over sulphuric acid and pounded. The powder, moistened with alcohol, was then extracted with water. The extract liquefied fibrin rapidly in the presence of dilute acids. Little attack of the fibrin occurred in neutral solution, so that these workers concluded that a pepsin-like enzyme was present. Mouton (1902) showed that *Amoeba* agglutinated *B. coli* and other organisms and utilised them for food by means of the secretion of the contractile vacuole ("grâce à la sécrétion de la vacuole pulsatile"); an enzyme was extracted from the cells which he regarded as tryptic in nature. Mesnil and Mouton (1903) grew *Paramecium aurelia* in a sterile medium for 15 days to one month, and then separated the organisms by an electrical method, collecting them at the cathode. The liquid caused rapid liquefaction of gelatin, its optimum activity being at the neutral point to litmus. It had a slight action on fibrin, and the authors concluded that the enzyme present resembled trypsin. Haughwout and Leon (1919) report observations on the ingestion of erythrocytes by species of *Pentatrichomonas*. Salle (1931) studied the metabolism of *Leishmania donovani*, and stated that this organism preferred carbohydrate to protein for purposes of energy, a utilisable carbohydrate in the medium in fact exerting a "sparing" action on the proteins of the medium, as has been reported for various bacteria.

Considerable attention of late has been paid to the enzymes of leucocytes. Jobling and Strause (1912) separated the polymorphonuclear leucocytes from inflammatory pus, washed in saline and dried with alcohol and ether. They found that the dried preparation gave an enzyme somewhat similar to trypsin which did not however attack peptone. Extracts from fresh undried leucocytes did however attack peptone. They concluded that two proteases were present. Willstätter, Bamann and Rohdewald (1929) made preparations from the leucocytes of horses, pigs and dogs. They found a weak tryptic action, catheptic action weaker still, and a strong ereptic action, and they noted a similarity in action between white blood cells and pancreatic gland extracts.

Willstätter and Bamann (1929) state that gastric mucosa contains two enzymes not secreted with the gastric juice, erepsin and a proteinase having optimum activity

at pH 3.5-4.0, which they called cathepsin. It is believed by these workers that both cathepsin and erepsin occur as leucocyte enzymes in gastric and intestinal mucosa, which is in agreement with histological observations on the accumulation of leucocytes in these tissues.

Kleinmann and Stern (1930) in studying leucocytes of the cow state that the spleen of this animal contains a proteinase hydrolysing native proteins such as serum albumin at pH 2.6-5.6, the optimum being at pH 4. Continued purification of the enzyme led to a narrower pH-range and a shift of the optimum to the acid side. Extracts of the spleen or of leucocytes behaved similarly. An ereptase showing optimum activity at pH 8 was also present. Grassman and Heyde (1930) studied the peptidases of blood serum. They found that normal serum contains little dipeptidase and considerable polypeptidase. Beef serum was two to three times, rabbit and sheep five times, and hog serum ten times as active as that of man or horse. Husfeldt (1931) carried out an extensive investigation of the proteolytic enzymes in the leucocytes of man. In a case of myeloid leukemia cathepsin, acting from pH 3-7, giving optimal cleavage of casein at pH 4.3, and edestin at 5.2 and a trypsin increasingly active from pH 4 onwards were found. The cathepsin resembles that of organs and represents the autolytic enzyme found in all animal cells. Glycerol extracts from leucocytes of normal blood contained considerable quantities of peptidases, showing greatest activity on tripeptides and less on tetra- and dipeptides.

VI. THE EFFECT OF THE COMPOSITION OF THE MEDIUM ON THE FORMATION OF ENZYMES.

The early workers found great variation in the activity of the enzyme obtainable according to the medium in which the bacteria were grown. Their outlook was often very anthropomorphic, sometimes almost attributing a conscious regulation to the cell. For example, Macfadyen (1892) states: "It is probable that in a highly nutrient medium like peptone gelatine the bacteria secrete very little of the enzyme. There is so much easily assimilated nutriment present that the bacteria are able to live without any 'struggle for existence' on their part. In such a rich soil the amount of food is far in excess of the requirements of the bacteria, and there is less necessity for an active secretion of their ferment." This teleological viewpoint is now largely discredited. It has been shown (Haines, 1932) that the gelatine liquefying enzyme gelatinase is formed in almost any medium provided certain salts are present, and it is as active when obtained from a broth culture as from a simple synthetic medium. Again, Quastel and Wooldridge (1927) working on the dehydrogenases showed that *B. coli* could bring about activation of a large number of substrates apparently by the same mechanism, including such substances as chlorates, entirely foreign to its normal environment. Moreover, the high energy exchange of bacteria is not dependent on the "need" of the organism, the oxygen uptake of a suspension of *B. coli* being practically the same whether the cells were viable or rendered incapable of multiplication by ultra-violet light (Cook and Stephenson, 1928). On the other hand, it is certain that the medium does exercise a profound influence

on the enzymes of the organism. Stephenson and Stickland (1932) found that the bacterial enzyme liberating molecular hydrogen from formates was only found when the organisms were grown in a medium containing formates. Yudkin (1932), who further investigated this point, came to the conclusion that it was not a question of selecting out those organisms which contained the enzyme from those which did not, but that a specific action by the substances in the medium took place on the organisms during growth. Beyond the undisputed fact that the medium does affect the proteolytic power of bacteria, little is known as to its exact influence, because so few studies have been carried out using carefully purified substances. The quantities required by bacteria for good growth are very small: for example, Friedlein (1928) found prolific growth of certain bacteria in a standard inorganic medium when the concentration of ammonium chloride as source of nitrogen was only 0.01 per cent., the minimum concentration of phosphate for good growth being 0.001 per cent. Moreover, Haines (1933) found that a concentration of 0.002 per cent. calcium chloride was sufficient to change the production of gelatinase from very poor or nil to good.

Early observations were those of Fermi already quoted, who found he could obtain a gelatin liquefying enzyme when *Micrococcus prodigiosus* and *B. pyocyaneus* were grown in a medium containing ammonium phosphate, certain salts and glycerol, but he concluded that it was not formed so readily as in broth. His results are open to criticism on the ground that in many of his media he used fermentable sugars which would give rise to large quantities of acid, and if these were not neutralised before testing for digestion no change would take place. In further work (1896) he grew fungi and certain bacteria in media containing only sugars or glycerin and which he claimed were free from nitrogen. *B. fluorescens* under these circumstances yielded a proteolytic enzyme which his methods of analysis showed contained no nitrogen. He states: "Einige auf stickstofffreien Nährböden entwickelte Mikroorganismen können ein Proteolin und Invertin bilden. Das Invertin und das proteolytische Enzym zeigten sich ebenfalls als stickstofffreie Körper. Es ist möglich, dass, wie die Zusammensetzung des Protoplasmas wechselt, auch jene der Enzyme variiert." Jordan (1906) obtained enzymes when asparagine was the source of nitrogen. Drummond (1914) working with an organism obtained from sludge digestions obtained a soluble enzyme when it was grown on gelatin peptone broth or peptone water, but none in sterile egg-albumin or an inorganic medium, and he concluded that peptone was essential for the formation of the enzyme with this organism. Diehl (1919) stated that filtrates from *B. pyocyaneus*, *B. prodigiosus*, and *B. subtilis*, grown in a medium containing diammonium phosphate as source of nitrogen, would not attack either gelatin or casein. If however glycine were the source of nitrogen gelatin was attacked but not casein, whereas with tyrosin casein was attacked but not gelatin. Since casein contains no glycine and gelatin no tyrosin, Diehl advanced the view that proteolytic enzymes are formed to correspond to different amino acids present in the medium, and will then attack these acids whether free or combined. Neither Merrill and Clark (1928), Wilson (1930) nor Haines (1932) could confirm this view.

Braun and Cahn-Bronner (1921, 1922) used a medium containing sodium chloride, potassium diphosphate and ammonium lactate. *B. para-typhosus* B grew well, and so did *B. pyocyaneus*, whilst Felix and Weils' *Proteus* did not. The first organism however decreased in virulence. Enzymes were obtained in the medium from *B. pyocyaneus*. Malfitano and Cutvire (1924) state that the presence of much iron in the medium causes lowering of the ability of *Aspergillus niger* to liquefy gelatin. Waksman and Lomanitz (1925) obtained breakdown of casein with various fungi growing in a synthetic medium containing this protein as the only source of nitrogen. Under these conditions *B. fluorescens* was unable to attack casein but could break down various amino acids, whilst *B. cereus* is stated to be able to decompose casein and other native (vegetable) proteins but not all the amino acids tried.

Merrill and Mansfield Clark (1928) made up the following synthetic medium using carefully purified substances:

Ammonium chloride 1 per cent.

Potassium dihydrogen phosphate 0.2 per cent.

Disodium phosphate 1 per cent.

Dextrose 1 per cent.

With the addition of salts of calcium and magnesium pH 7.0.

They found that the gelatin liquefying enzyme was produced in this medium when salts of calcium and magnesium were present, but not in their absence, although adequate growth occurred in the absence of these salts. The fundamental importance of these salts probably explains the conflicting results of earlier workers and the whole question of protein breakdown requires reinvestigation in this light. Wilson (1930) criticised Merrill and Clark's findings on the ground that poor growth occurred in the absence of the above salts, and concluded that they had no specific effect on enzyme production apart from their growth-stimulating properties. Haines (1932, 1933) extended the observations of Merrill and Clark to a variety of organisms and media, and confirmed their findings. In the foregoing medium with the addition of magnesium chloride excellent growth takes place but little or no gelatinase is formed with most organisms. In the presence of calcium chloride alone, on the other hand, although growth is definitely poorer than in the presence of magnesium chloride, good gelatinase production occurs. Salts of calcium would seem, therefore, to have a specific effect on enzyme production apart from any stimulation of growth. A similar effect is obtained with a variety of sources of nitrogen and persists on repeated subculture of the organism. Whether the enzyme "gelatinase," causing liquefaction of gelatine, is a separate enzyme, or liquefaction is the work of a proteinase, is not certain. The available data would seem to point to the view that it is a distinct enzyme (Northrop, 1931). There is, however, evidence that a similar result follows with regard to the enzyme attacking casein in the first subculture in the synthetic medium; it has yet to be shown that it persists in subculture. Whether this effect is merely quantitative in nature, that is, increasing in amount enzymes which are otherwise formed slightly, by some

action as on the permeability of the cell wall (Stephenson, 1930), or qualitative, so that some amount of calcium is absolutely essential for the formation of the enzyme, none being obtained in its entire absence, is not yet certain. The results so far obtained point to a quantitative interpretation (Haines, 1932), but the amounts of calcium required are so small that certainty is difficult. The mechanism of the action of these salts is at present unknown and awaits further research. Whatever may be the ultimate truth about this point, if a suitable synthetic medium can be devised in which an active enzyme is produced, it would seem to offer a better means of obtaining a "pure" enzyme preparation than meat extracts or peptone, from which so much protein impurity has to be removed by precipitation methods.

Another factor influencing protein breakdown is the amount of available carbohydrate in the medium; it has been investigated in detail by Kendall and co-workers (Kendall and Walker, 1915). A summary of the literature may be found in De Bord (1923). Similar results have been reported for fungi by Waksman (1917) and for Protozoa by Salle (1931). Apparently when a fermentable carbohydrate is present some organisms under certain conditions utilise this as a source of energy in preference to attacking the protein, and no proteolytic enzyme is formed. Care must be taken to distinguish a true "protein-sparing" from effects due to the change in hydrogen-ion concentration in the medium by reason of the sugar fermentation, and to allow for the possible inhibitory effects of acids formed. Kendall's views, which are in some measure a return to the view that the organism only forms the enzyme in response to need, have been criticised by Berman and Rettger (1916).

Recent work on the effect of the medium on the formation of fungus proteases has been carried out by the Japanese experimenter Wada (1930). He grew *Penicillium glaucum* in a solution containing sodium chloride, monopotassium phosphate, magnesium sulphate, ammonium carbonate and glycerin. After 7-11 days at 20-32° C. the mycelium was filtered off and well washed. It was then ground with quartz sand and pressed at 150 atmospheres. A slightly acid, lightly coloured opalescent liquid resulted. Experiments were also carried out with the culture fluid. In the protein-free medium pepsin- and trypsin-like enzymes, erepsin and a deamidase were obtained from the mycelium whilst the presence of a pepsin-like enzyme was shown in the culture fluid. Comparison with the enzymes obtained after growth in a medium containing cane sugar, meat extract and peptone showed, however, that the production of the pepsin- and trypsin-like enzymes was restricted in the protein-free medium, and Wada concludes: "Ob diese Verminderung einer geringeren Fermentbildung oder einer weniger aktiven Modifikation des Ferments entspricht, muss natürlich weiteren Untersuchungen vorbehalten bleiben."

VII. RÉSUMÉ OF METHODS.

The formation of exo-enzymes may be shown in the case of bacteria by centrifuging off the organisms, with or without filtration, through a suitable filter candle or Seitz asbestos filter, and incubating with the appropriate substrate at the required

pH. Further concentration and purification may be carried out by evaporation *in vacuo* at low temperatures (Hladik, 1910), fractional precipitation with ammonium sulphate (London and Pakhotina, 1917), precipitation, dialysis and re-precipitation with alcohol (Münter, 1910), adsorption on alumina or kaolin and fractional elution with suitable buffers of selected pH (Willstätter, 1928; Hopkins, 1930), dialysis (Harden and Young, 1906; Walter, 1917), and extracting with glycerine (Macfadyen, 1892).

With moulds and actinomycetes the mycelium may be filtered off through paper and suitable methods of concentration applied to the filtrate. For the preparation of endo-enzymes of bacteria, autolysis may be used, alternative freezing and thawing (Young, 1929), grinding in an agate mortar (Stevens and West, 1922, *a, b*), or lysis with bile (Avery and Cullen, 1923). For yeasts and fungi the "acetone-dauerhefe" process employed by Dox (1913), combined grinding and extraction of juice by high pressure (Buchner, 1898), and low temperatures (Rowland, 1901), are available, after which further precipitation may be carried out. Halderer (1909, 1910, 1912) investigated the conditions influencing filtration of enzymes through porcelain filters. Two interesting recent methods of following protein hydrolysis are by Krebs (1930) and Gates (1930). The former dissolved the protein in bicarbonate solution in the cup of a Warburg manometer in the presence of 5 per cent. carbon dioxide. As hydrolysis proceeds the carbon dioxide tension changes and may be read off on the manometer. Gates measured the reduction in density of exposed photographic film in a photometer, progressive proteolysis of the gelatin leading to release of silver. Viscometric methods are well adapted to the study of breakdown of gelatin (von Gröer, 1912; Haines, 1933). A nephelometric method of following proteolysis has been developed by Rona and Kleinmann (1928). Foreman and Graham-Smith (1928) worked out titration methods for following the changes in meat broth during growth of *Staphylococcus aureus*.

VIII. THE PRESENT POSITION.

Based on the foregoing survey of the literature, the present state of our knowledge of microbial proteolytic enzymes would seem to be as follows.

Bacteria will not initiate breakdown of native proteins unless sufficient simple nitrogenous material is present for them to multiply vigorously and elaborate an enzyme. Fungi, on the other hand, apparently may do so, and possibly also Protozoa, these points however requiring further investigation. Even when a good supply of nutriment is available, only a few bacteria elaborate enzymes capable of attacking native proteins, and this attack takes place more easily if the protein is previously denatured by heat or lactic acid. Breakdown of proteins is in the first instance of the nature of simple hydrolysis: this may be brought about by aerobes or anaerobes. After that the course of further decomposition would appear to depend largely on the oxidation-reduction potential of the medium. If conditions are aerobic, evil-smelling substances containing the —SH group are not liberated because they undergo oxidation or further decomposition. Indol is produced aerobically, but

can also be further broken down by some aerobes. The evil-smelling amines are chiefly liberated in a medium of high reducing intensity. Thus these substances are set free in greatest quantity in conditions under which only the strict anaerobes grow, and are regarded by many as characteristic products of anaerobic growth. The early workers are therefore in this sense justified in their statement that it is the strict anaerobes which are responsible for "stinking" putrefaction. There would appear to be no grounds for assuming that the mechanism of proteolysis is in the preliminary breakdown different in the two cases. Although quantitative comparisons are lacking, probably a larger proportion of the anaerobes than of the aerobes synthesise active proteinases. Possibly the proteinases of the anaerobes may be on the whole more active than those of the aerobes, but impressions gathered from qualitative experiments may be misleading since the incomplete oxidation of the split products leads to the liberation of less energy available for the micro-organism. Consequently large quantities of material are quickly decomposed when rapid multiplication of anaerobes takes place. In the present state of our knowledge it is probably better to regard the distinction between "putrefactive" and "non-putrefactive" bacteria as one of degree and not one of kind. That is to say, the strict anaerobes produce in greatest quantity the compounds which, by definition, are characteristic of true putrefaction, but aerobes may produce some of these products. In particular, the metabolic products of a facultative anaerobe may be modified by the conditions under which it is grown, *e.g.* haemolytic streptococci which are normally regarded as non- H_2S producers have been found to yield some H_2S by anaerobic incubation on a medium containing cystine. Most bacteria possess enzymes capable of splitting peptides and substances of the complexity of peptone. The enzymes of some micro-organisms are synthesised when they are grown in very simple media containing no protein, but the amount or activity of the enzyme so formed is influenced by various constituents of the medium in a manner not yet understood. Broadly speaking the majority of bacteria elaborate an enzyme which is tryptic in nature, acting on the alkaline side of neutrality, whereas possibly in fungi and more definitely in Protozoa there is a transition to the state in which pepsin-like enzymes acting in acid medium are found. Here again however further investigation by modern methods is desirable. Those cases which have been investigated in detail by modern methods afford evidence for the view that bacterial proteases are mixtures of two or more separate enzymes—in yeast a proteinase, a polypeptidase and a dipeptidase, in *B. fluorescens liquefaciens* a proteinase, an enzyme splitting peptone and a dipeptidase. Whether the enzyme liquefying gelatin is to be considered a separate system is undecided. Evidence obtained by immunological methods (specific antibody formation) would seem to point to the view that enzymes from two species of bacteria which bring about a similar change in the same substrate are not identical, but this is open to the criticism that the proteins causing specific antibody formation are present as impurities, or are merely colloidal carriers of the same active group. The question whether we are to regard "pure" enzyme preparations, *e.g.* crystalline pepsin, as a "pure" enzyme in the sense in which we may have pure sodium chloride, or to regard the protein as adventitious

and replaceable by any other suitable colloidal carrier, must be left until more is known of the nature of the active group. According to Waldschmidt-Leitz (1931) dipeptidase attaches itself to its substrate by means of the NH_2 group, and the "haptophore" or active group is an aldehyde or keto group (Euler and Josephson, 1923). Summaries of these recent views of the nature and action of proteolytic enzymes may be found in English by Waldschmidt-Leitz (1931) and Willstätter (1927), and a general discussion of the specificity of proteinases and peptidases by Haldane (1930, p. 110).

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THE SYMPLASMIC STATE OF THE TISSUES OF THE ANIMAL BODY

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CONTENTS.

	PAGE
I. Introduction	263
II. Discovery of the cell and foundation of the cell theory	264
III. Discovery of protoplasm and first reform of the cell theory	266
IV. Discovery of plasmodia and syncytia; the energids of Sachs	266
V. First theories of the origin of ground substances and fibrils	268
VI. Ground substances as exoplasms; formative secretions	269
VII. Protoplasmic networks as a basis for ground substances	273
VIII. Ground substances of bone and dentine	275
IX. Further work on the exoplasm theory	276
X. Opponents of the cell state theory: Heidenhain, Rohde, and criticism of their opinions	277
XI. Extracellular protoplasm; criticism of the energid theory; plasmatology	281
XII. Terminology and classification of symplasmic structures; a new definition of animal tissue	285
XIII. Extreme standpoints: opponents of the symplasmic theory and criticism of their views	288
XIV. Examples of non-cellular structures and tissues	291
XV. Terminology of bioplasms	293
XVI. Summary	294
References	295

I. INTRODUCTION.

THE older histology, represented by most text-books of to-day, favoured the idea that the body of an animal consists of cells and defined tissues as complexes of well-delimited, living cells composed of caryoplasm and cytoplasm, and of their products the "formed secretions." The whole body of the animal was accordingly a "cell state," the life of which was a result of the life of its components, the cells. There is, however, a tendency in recent histology to replace this by another idea, according to which the body of an animal is a unit in which the cytoplasm, containing nuclei, is continuous throughout.

The cytoplasm, of which the whole body is composed, is not uniform; on the contrary it can be differentiated into the following three varieties: (1) endoplasm

(protoplasm proper), representing the undifferentiated, original cytoplasm, forming the cell bodies plus the prolongations of the cells, and conforming in most cases to the "cells" of the older histology; (2) ectoplasm or exoplasm (the "periplast" of Huxley, 1853) which is usually denser than the endoplasm and forms the outer layers of many cells; it separates the endoplasms (*i.e.* the "cells" of the older histology) and can accumulate according to the views of modern histology in the form of "ground substances" between cells, or as a "cuticular substance" on the surface of cells¹; (3) functional structures, fibrous formations (*i.e.* fibres or bundles), into which both endoplasm and ectoplasm can occasionally differentiate and which occur in the form of tonofibrils, myofibrils and neurofibrils.

The original cytoplasm of the embryonic body undergoes partial differentiation during development, either into ectoplasm or directly into the functional structures, the rest remaining undifferentiated in the form of endoplasm. There are undoubtedly also retrograde differentiations (de-differentiations), but up to the present we have very little information concerning these. In addition to the living constituents, non-living (metaplastic) substances also take part in the structure of the tissues, but, according to the view of the new histology, the former only impregnate the latter.

The older histology, which regarded the cells as the only living components of the body, did not realise all these changes of the original protoplasm and considered everything situated between the cells and separating them from each other (cell membranes, septa, cement substances and ground substances) as secretions without any living activity, by means of which the cells are held together. In the same way it considered the greater part of these structures, for example connective tissue fibres, as dead formations (chiefly coagulations), the arrangement of which depended on the cells.

In the title of this article I have employed the term "symplasmic states" for all cases in which the cytoplasm (or the formed bioplasm, as I shall call it below) in the body of the animal is continuous. By symplasmic state I understand also cases in which the cells are connected with each other by means of cytodesmata, cases in which exoplasmic ground substances are situated between the cells and such cases in which the cytoplasm forms large structures and continuous masses and layers.

II. DISCOVERY OF THE CELL AND FOUNDATION OF THE CELL THEORY.

To understand how the ideas concerning the structure of the animal body originated and were discussed it is necessary to take into consideration the ideas of plant anatomists which very often preceded and influenced those of zoologists.

At the beginning of the nineteenth century there was a tendency to explain all elementary constituents of the plant body as cells. It was known that cells alone were present at the beginning of the development of a plant and that the lowest plants consisted only of cells (Turpin, 1826; Meyen, 1830). As early as 1806 Treviranus showed that the tracheae in plants originated by confluence of the

¹ Or it may represent the substrate of this substance.

cavities of cells (he did not recognise cell walls), and in 1831 Mohl demonstrated the same thing more accurately. Unger (1838) showed that milk vessels of plants (at least some of them) developed in a similar way, and thus it was shown that the whole plant had its origin in cells¹. Many authors attribute this knowledge to Schleiden, but this is not correct; we owe to Schleiden (1838) only the erroneous idea of the origin of plant cells from cell nuclei².

At the beginning of the microscopic study of the structure of the animal body the existence of elementary constituents of the same sort was foreseen. Albrecht v. Haller (1757) saw them in fibres, other authors in fine granules which they had observed in squashed tissues with their imperfect microscopes, or both of these elements were suggested. Milne Edwards described fine "molecules" in various animal tissues up to 1823, and Dutrochet (1824) thought that such molecules developed into vesicular formations and he put forward a kind of cellular theory³. Later Purkinje with his pupils (Valentin, etc.), and Johannes Müller and his pupil Henle, discovered larger formations in animal tissues comparable with cells and even corpuscles similar to the cell nuclei which Robert Brown described in plant cells in 1831. The idea that these "granules" containing nuclei corresponded with the plant cells and cell nuclei was first definitely and clearly put forward by Purkinje in 1837, who attributed to them the same physiological significance⁴. But he did not follow this idea far enough and did not systematically collect the facts already known to him, and perhaps for this reason credit for the foundation of the theory of the conformity in the structure of the body of plants and animals is generally given to Theodor Schwann, who dealt with this theme in a detailed paper (1839). Purkinje mentioned "granules" without a special membrane, whilst Schwann described vesicles formed by the cell membrane, "cells," and therefore their descriptions of animal tissues do not quite agree.

As the outcome great emphasis was laid on "cells" in animals just as was earlier the case in plants. Animal tissues were explained as complexes of cells and their derivatives. It was shown later that "cells" were the first structures from which animals developed and that the components of the animal body, which did not correspond with cells, developed from the cells or were the products of the latter (Reichert, 1845). Later it was realised (Siebold, 1845) that the simplest animals—the Protozoa of to-day—represent free-living cells.

Under Schwann's influence the idea of the animal cell as a structure isolated and surrounded by a membrane originated and was maintained. Cells, both of plants and animals, were understood as mere vesicles, even after their living contents (protoplasm) had been discovered, and even now the idea that cells are independent and isolated wholes is maintained by many biologists. Many recent theories and hypotheses, especially in physical chemistry, are based on the idea that animal cells are independent structures surrounded by a limiting layer.

Before it was possible to describe cells as elementary constituents of the animal

¹ Cf. Sachs (1875).

² Cf. Studnička (1933 b).

³ Cf. Studnička (1932) and Florian (1932).

⁴ Cf. Studnička (1927).

body it was necessary to correlate with cells all the constituents of the tissues which were different from cells, as had earlier been done in plants. Accordingly we see that before the origin of the cell theory Valentin (1832) maintained that muscle fibres originate from granules which arrange themselves into rows and then join. Most probably this idea was also known to his teacher, Purkinje (1839, 1840), who considered "fibres" as formations occurring in the animal body in addition to "granules." Schwann accepted Valentin's interpretation of the origin of muscle fibres in his book (1839) and adopted a similar interpretation of the origin of nerves and capillaries as formations originating by the confluence of cells. Collagenous fibres, on the contrary, originated according to Schwann by cell splitting and elastic fibres represented transformed and elongated cells.

The interpretations of Valentin and of Schwann were maintained for a long time. Kölliker, for example, speaks (1852) of "higher elementary constituents," under which heading he understood structures originating by the confluence of cells; he already knew of cell nets (enamel pulp) which he includes in this group.

III. DISCOVERY OF PROTOPLASM AND FIRST REFORM OF THE CELL THEORY.

The discovery of the living substance represented a great advance in the interpretation of the animal cell which, up to that time, under Schwann's influence, was considered as what we now call the cell membrane. Purkinje (1839) already knew this substance and attached to it the term "protoplasm" for the "granules" composing the embryonic body. Later on Mohl (1846) employed this term for the gelatinous substance of plant cells, known to the older botanists as "gum" or "mucus." Remak (1855) introduced Purkinje's term "protoplasm" into zoology, and Max Schultze (1861) defined the cell as a "mass of protoplasm containing a nucleus." It was exactly what Purkinje (1837) had called the "granule with central nucleus."

Since that time cells have been regarded as "masses," but even now their individuality is emphasised. The "higher elementary constituents" of Kölliker are considered only as exceptions to this rule. Later, as will be stated below, additional exceptions were discovered, but the idea of the cell plasm as an isolated formation has taken such strong root that Verworn up to 1895 was able to maintain that "there was no living substance which could not be differentiated into cells." But this was not substantiated. Already in the 'nineties many exceptions to the classical cellular theory were known and very soon afterwards it was observed that there existed quite extracellular living components in the animal body, *i.e.* the ground substances, which were previously interpreted as mere cell products. Briefly, it was discovered that the structure of the animal body was much more complicated than that of the plant.

IV. DISCOVERY OF PLASMODIA AND SYNCYTIA; THE ENERGIDS OF SACHS.

The botanist De Bary observed (1859) the formation of "plasmodia" by confluence of small amoeboid developmental stages in Myxomycetes. Such a plasmodium represents a non-cellular organism and produces sporangia with

spores. Haeckel (1872) called attention to the fact that the ectoderm of the *Calcispongia* is composed of a continuous layer of protoplasm in which the nuclei are dispersed. He designated this non-cellular tissue as "syncytium." We thus see that from the start two terms have been employed for nearly one and the same structure, the term "plasmodium" being the older; later Haeckel's term was preferred.

In 1885 Sedgwick called attention to such "syncytia" in a paper in which he showed that the germ layers of *Peripatus capensis* develop into tissues always forming continuous layers of protoplasm. But embryologists already recognised a segmentation without any division into cells in the "superficial" segmentation of arthropod eggs. In this case the nucleus undergoes a division several times, the nuclei so formed migrate to the cell surface and only afterwards does the proper segmentation take place. In *Peripatus* and in the cases mentioned below (pp. 280, 288) no cells differentiate even afterwards. The theoretical significance of these "non-cellular" states or "syncytia" was only later realised.

Whitman (1893) was the first to put forward objections to the "traditional cell standpoint" of those days in an article entitled "The inadequacy of the cell theory of development." Apart from other statements, he showed that the nephrostomes of some species of Annelida are composed of cells, but in other species they consist of a mass of protoplasm undifferentiated into cells, and he wrote: "The nephrostome is a nephrostome all the same whether it consist of one cell, two cells or many cells... So far as homology is concerned the existence of cells may be ignored." He refers to De Bary's (1879) and Rauber's (1883) "organism standpoint" as representing the opposition against the "cell standpoint" of contemporary biologists. But there is a difference between his and De Bary's view. The latter had in mind a whole, *i.e.* the organism, composed of cells, to which the cells were subordinated ("Die Pflanze bildet Zellen, nicht die Zelle bildet die Pflanzen" (De Bary, 1879)). He paid no regard to the syncytia, but both of them (Whitman and De Bary) emphasise that the organism represents a whole.

Whitman was followed by Sedgwick (1895) in a paper with a very similar title: "On the inadequacy of the cell theory of development." Here the author called attention to the "syncytial state" of the mesenchyme, which was, in his day, interpreted as consisting of well-delimited cells, and also to the development of nerve fibres by the confluence of a series of cells (a view which was proved later not to be correct). The views of both Whitman and Sedgwick were opposed to the cell theory, but they arrived at no definite conclusions. This was done by Delage (1896) and Labbé (1897). In their opinion "syncytia" represented the original state, whilst differentiation into cells was regarded as secondary in phylogeny and in ontogeny. For example, Labbé suggested the possibility of the origin of Metazoa from multinuclear Protozoa (such as *Opalina*) by division of the cytoplasm into cells. Both authors refer to "superficial segmentation" in which cells are only formed as an addition after nuclei have increased in number.

A little earlier the botanist Sachs (1892) made an attempt to solve the problem of "syncytia" from a different standpoint. His object was to explain the structure

of the large bodies of algae belonging to the order Siphonae (*Caulerpa*, etc.) which contain many nuclei in the cytoplasm, there being no differentiation into cells. He distinguished "cells" from "energids," the latter being a region of cytoplasm ruled over by a single nucleus. In his opinion there may exist "cells" containing a single nucleus (here "cell" and "energid" coincide), or "cells" containing many nuclei, in which there are as many "energids" as nuclei.

It is clear that the energid theory brought great support to a cell theory endangered by the discovery of syncytia. It was possible to suggest the existence of "energids" in all cases where nuclei could be observed; the uni-nucleated "individual" cell represented simply just an "individual" energid. The question was whether all cases could be explained in this manner; recently an answer has been provided which will be given below (p. 284).

Whilst the energid theory was well received by biologists, the "syncytium theory" of Sedgwick, Whitman, Delage and Labbé met with little support. The main objection against the latter was the fact that most tissues are actually composed of well-differentiated cells and that in numerous tissues the cells are distinctly separated even by non-living septa, cement substances and ground substances (such, at least was the opinion of the biologist of those days)¹. Protoplasmic connections between the cells of animal tissues, it is true, had been observed², but not everywhere, and so the theory of "individual cells" forming the elementary constituents of the adult animal body held the field. On the other hand, it was possible to object to the cases of "superficial segmentation" (see p. 267) put forward by the opponents of the cell theory, that we have here to do with quite exceptional conditions caused by the presence of enormous quantities of yolk in egg cells. The references to the "Inadequacy of the cell theory" were then almost generally rejected, but the objections to the correctness of the cell theory were repeated in a new form in the last decades of this century (cf. p. 279) when the conditions changed considerably: it was now possible to refer to the ground substances which represented the main obstacle to the solution of this problem.

I shall therefore leave the discussion of the differentiation of protoplasm into cells and syncytia and review the investigations of the ground substances.

V. FIRST THEORIES OF THE ORIGIN OF GROUND SUBSTANCES AND FIBRILS.

In 1861 Max Schultze, who returned to Purkinje's idea of 1837 and defined the cell as a "mass" of protoplasm, had already expressed the opinion that developing connective tissue in animals was originally composed of naked cells continuous with each other and that connective tissue fibres originated from these.

¹ It was then supposed that the cells of epithelia are also separated from each other by thin layers of "cement substance." It was only later that histologists observed intercellular spaces, mostly quite narrow, between these cells.

² In 1864 Max Schultze discovered "spines" on the surface of the cells of stratified epithelia. Bizzozero (1871) showed that the extremities of these spines are connected with each other. The discovery of such cell bridges completed the cell theory; it showed that the cells need not represent isolated individuals, but otherwise it did not endanger the cell theory. I shall therefore not deal in detail with these discoveries concerning cell connections.

In this way he indicated that ground substance originated from protoplasm. His pupil Landois (1866) expressed this view precisely, and in 1872 another pupil of Max Schultze, Boll, described the formation of connective tissue fibres in protoplasm more in detail. Beale (1861) and Brücke (1861) also spoke of the origin of ground substance by transformation of protoplasm. Max Schultze and Brücke did not mention the question of the vitality of ground substance, while Beale, on the contrary, regarded ground substance as a "formed matter" (*i.e.* non-living) in contradistinction to "germinal matter" (*i.e.* cells). He simply followed Virchow's (1858) idea which regarded cells alone as living, the rest of the animal body (their products) being subject to their influence¹. Even Boll, who described the formation of connective tissue fibres in cell protoplasm, regarded the fibres as cell products (like fat, starch, etc.) and so accepted Virchow's opinion on this point.

Quite different views were expressed by Heitzmann and Stricker. The former suggested (1873) the presence of a fine protoplasmic network in the ground substance and regarded the latter as living. Stricker (1883), who also regarded ground substance as living, did not, on the other hand, attach any importance to such a network. His ideas were rather fantastic; for example, in his opinion the cells by the conjunction of which the ground substance originated could arise once more in the latter². He also suggested movements of the ground substance as a sign of its vitality. This has never been seen by anybody else, since the vitality of ground substance is shown mainly in its "formative life" (see below, p. 276). Among pathologists, the Russian worker Lukjanow (1897) regarded ground substances as living and accordingly as a product of protoplasmic transformation. These theories aroused little confidence amongst contemporaries.

The correct interpretation of the significance of ground substances was first made possible in the 'nineties by the study of their genesis, by comparison with the solid layers ("exoplasm") differentiated on the cell surface, by observation of connections of cells with their networks, and finally by studies of the purposeful structure of fully developed ground substances. As a result the morphological significance of ground substances came to be regarded in a different light. But even to-day many workers regard ground substances as mere accumulations of connective tissue fibres (see further, below, p. 290) and do not take into consideration the fact that ground substances may assume different arrangements (such as frameworks, systems of lamellae or compact masses).

VI. GROUND SUBSTANCES AS EXOPLASMS; FORMATIVE SECRETIONS.

We will now deal with theories of the development of ground substances as a whole; theories of the genesis of fibres will not be discussed at present. We will begin our history of the newer observations on the genesis of ground substance with Retterer (1896), in whose opinion the mesenchyme which forms a "syncytium"

¹ He employs the term *Einflussphaeren* for the designation of areas of ground substance ruled over by single cells.

² The same idea reappears later in the theories of Grawitz.

differentiates partly into a "chromophil plasm" (*i.e.* cells) and a "hyaloplasm"¹ (*i.e.* ground substance).

In 1899, Hansen employed the term "exoplasm."² He showed by very accurate observations that the capsules situated on the periphery of the cells in the annulus fibrosus of intervertebral discs (*i.e.* in somewhat differentiated tissue) originate by transformation of the protoplasm. The protoplasm which does not undergo this transformation represents the endoplasm³ (Figs. 1, 2). Further, he described the formation of collagenous and elastic fibres in both the original protoplasm (endoplasm) and in the capsules (exoplasm) of the cartilage cells. In his opinion in later stages the fibres are formed only in the exoplasm, and the continuous ground

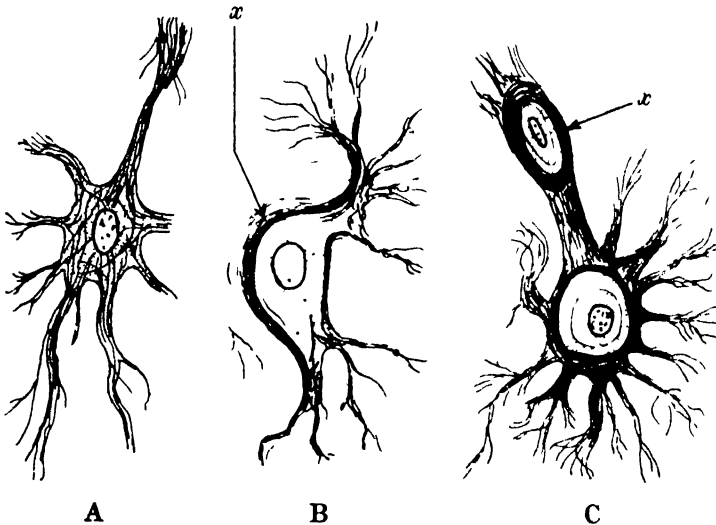


Fig. 1. Development of capsules in mammalian intervertebral cartilage at the expense of exoplasm. A, a cell without differentiation into exoplasm and endoplasm. B, a cell in which the fibrils are concentrated in the exoplasm (x). C, two young cartilage cells with capsules (x). (After Hansen, 1899.)

substance of the cartilage originates by the confluence of the exoplasm of different cells⁴.

Mall (1902) follows next with his paper on the "connective tissue syncytium." In accordance with Sedgwick's view, he regarded mesenchyme as a syncytium, but held that it constituted a syncytium in which the protoplasm is differentiated partly

¹ The term "hyaloplasm" had already been adopted for the designation of the homogeneous component of cytoplasm by Hanstein (1880).

² This term which has not been abandoned since, was first introduced into biology by Haeckel (1872) in a paper on the Calcispongia. Renaut (1886) employed the term for the designation of the cell membranes and the capsules in cartilage, whilst the cytoplasm of the cells was designated as endoplasm. In 1898 I called attention to the analogy between exoplasm (of the notochord and in epidermal cells) differentiated into fibrils and ground substance.

³ In 1853, Huxley employed the term "endoplast" for the designation of the protoplasmic cell body. His "perioplast" corresponds to a part of our "exoplasm."

⁴ In 1925 I found a similar structure in the elastic cartilage of the human epiglottis. In this case too collagenous fibrils originate in exoplasmic capsules (cf. Fig. 12 C, p. 286).

into a perinuclear endoplasm (the cells) and partly into exoplasm. The latter "splits" into collagenous or elastic fibres. In cartilage and bone he distinguished in addition the proper ground substance which he interpreted as a secretion.

The question now arose as to what was the true nature of exoplasm and whether it produced the fibres or split into them. In this connection I come to the discussion of my own papers.

In 1897 I described observations on the cartilage of cyclostomes (*Myxine*, *Petromyzon*) and on the chorda dorsalis of fishes (1897 *b*). In the former there are distinct stratified capsules, in the latter, especially in teleosts, one can observe very broad exoplasm (of Renault) containing plasmofibres (Figs. 2, 3 A, B). In 1899,

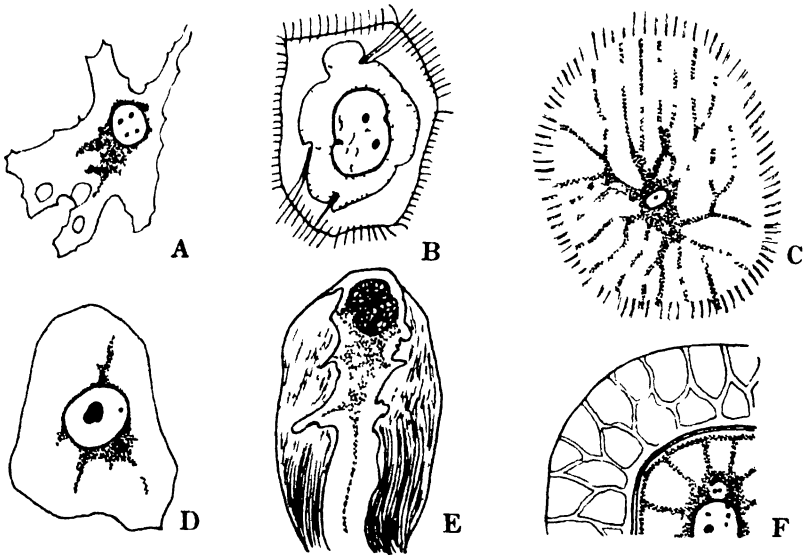


Fig 2 Various cases of cells with a broad undifferentiated or differentiated (fibrillar) exoplasm (crusta) A, a fibrocyte from the subcutaneous connective tissue of a mammal, with a lamellar exoplasm (after Jasswoin, 1928) B, a cell from the epithelium of the lip of *Chamaera monstrosa*; exoplasm with bundles of tonofibrils (after Studnička, 1909) C, a cell from the "septum" of the notochord of *Esox lucius*, the exoplasm with canals into which endoplasmic processes penetrate (after Studnička, 1931) D, a "Kolbenzelle" from the epidermis of *Ophidium barbatum* (after Studnička, 1909) E, a "Kolbenzelle" from the epidermis of *Petromyzon marinus* with a fibrillar differentiated exoplasm (after Studnička, 1909) F, a cell with a thin cell membrane, a capsule and a pericellular area, from the elastic cartilage of the human epiglottis (according to my observations, 1925) (In all figures endoplasm finely dotted)

I also studied other objects: the epithelium of the epidermis of fishes (with distinct exoplasm on the cells closely attached to each other), the "reticular" epithelium of the stellate reticulum of the enamel organ in the dentine teeth and the tissue comparable with the latter in the horny teeth of the Cyclostomata (Fig. 3 C, D). By comparison of all these tissues I became convinced (1902 *b*) that there was no sharp distinction between the individual exoplasm of the cells of the chorda or epithelium and the continuous exoplasm of the ground substances.

I supported my conclusions not only by a comparison of the fully developed tissues but also by a study of the histogenesis of the cartilage and the connective

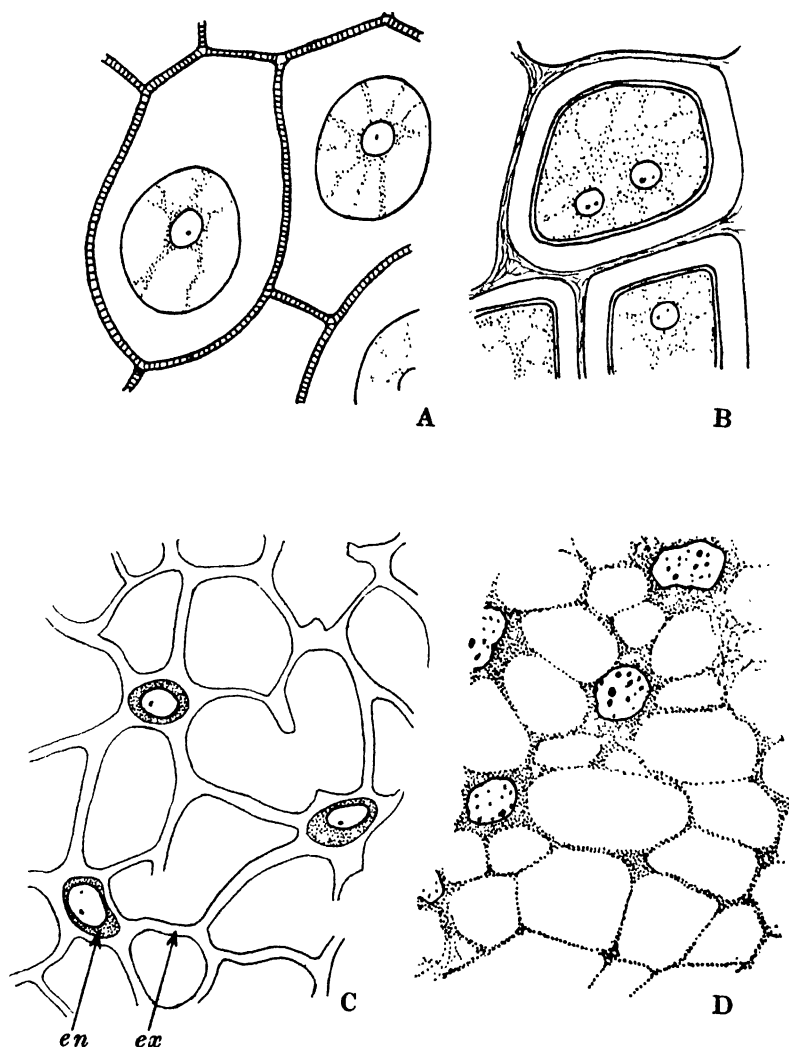


Fig. 3. A, epidermoid cells from the notochord of *Cyprinus auratus* with a broad homogeneous exoplasm, compared with B, cells of the parenchymatous cartilage from the tail fin of *Petromyzon* with capsular structures. C, reticular epithelium composed of cells with a differentiated exoplasm (*ex*), the endoplasm (*en*) being present in the neighbourhood of the nuclei only. From the surface of the spine of the dorsal fin of a foetus of *Spinax niger*. D, reticular epithelium composed of soft cells from the horny teeth of *Petromyzon planeri* (after Studnička, 1897, 1903, 1909 and unpublished).

tissue, regarding (in accordance with Mall) the primitive reticular mesenchyme as the first developmental stage. I called (1902 *b*) attention to the analogy between "plasmofibres" (*i.e.* protoplasmic fibres) in the tissues composed of well-delimited

cells and connective tissue fibres in the tissues characterised by a continuous exoplasm (cartilage, fibrous connective tissue). In a detailed study (1903) I tried to give further proofs for my opinion. In another paper (1903 *b*) I published schematic figures of the genesis of the tissues mentioned, to show the analogies existing among them (especially among the fibrous formations), and to make clear the relations of ground substances and fibrous structures to the "cells" (Fig. 4). As early as 1902 I regarded the stellate reticulum of the enamel organ and reticular epithelium generally as analogous to the reticular stages of mesenchyme.

I expressed the opinion that in the course of its transformation into a fibrous tissue the cytoplasm of mesenchyme in an advanced state of its development differentiates into two distinct kinds of protoplasm¹: the inner, perinuclear protoplasm remains almost undifferentiated (this I have designated (with Hansen) as "endoplasm"), whilst the superficial protoplasm differentiates into a denser "exoplasm." Differentiation may proceed so far that the two kinds of protoplasm become clearly delimited from one another. We may then distinguish a "whole cell" (*Gesamtzelle*) comprising both the endoplasm and the exoplasm belonging to it, and a "cell" strictly speaking corresponding to the endoplasm only² (see Fig. 4 A). I have shown that the whole cells of the connective tissue may be arranged in rows or layers (in accordance with Laguesse's views published later, 1914). In addition, I called attention to the fact that very different structures are sometimes interpreted as "cells"; in epithelia and notochord we have to do with "whole cells," whilst in connective tissue and in cartilage the "cells" of authors correspond to the "endoplasm" cell.

This "exoplasm theory" (*i.e.* the theory of the exoplasmic origin of ground substances representing one of the transformation theories (see below)) does not completely explain all cases of the histogenesis of ground substances. Sometimes, in addition to the transformation of protoplasm into exoplasm, the exoplasm becomes impregnated by secretions, for which I employed the term "formative" secretions (*Bausekrete*) in 1911 *b*; this is the case in the hard ground substances of cartilage, bone and dentine. Thus the formative substance in these "formative tissues" (*Baugewebe*) becomes a combination of exoplasm, fibres and secretions. (The "cuticular substance" of the invertebrate cuticula is an analogue of the "ground substance.")

VII. PROTOPLASMIC NETWORKS AS A BASIS FOR GROUND SUBSTANCES.

It has been observed that ground substance can originate not only by the transformation of the peripheral protoplasm of the cell bodies, but also by a similar transformation of protoplasmic *networks* formed by the prolongations of cells.

¹ In this connection I must call attention to the important difference between my opinion and that of Retterer (1896); the latter has also supposed a differentiation of the protoplasm of mesenchyme in two directions, but in his opinion mesenchyme represents a completely continuous syncytium, the intercellular spaces of authors representing the "hyaloplasm."

² As early as 1853 Huxley employed the term "the whole of the cell" for the designation of a unit composed of the "periplast" (the cell wall) and "endoplast" (the protoplasm with the nucleus). He does not speak of the origin of the periplast by the transformation of the endoplast. Landois (1866) also speaks of *Gesammtzellen*.

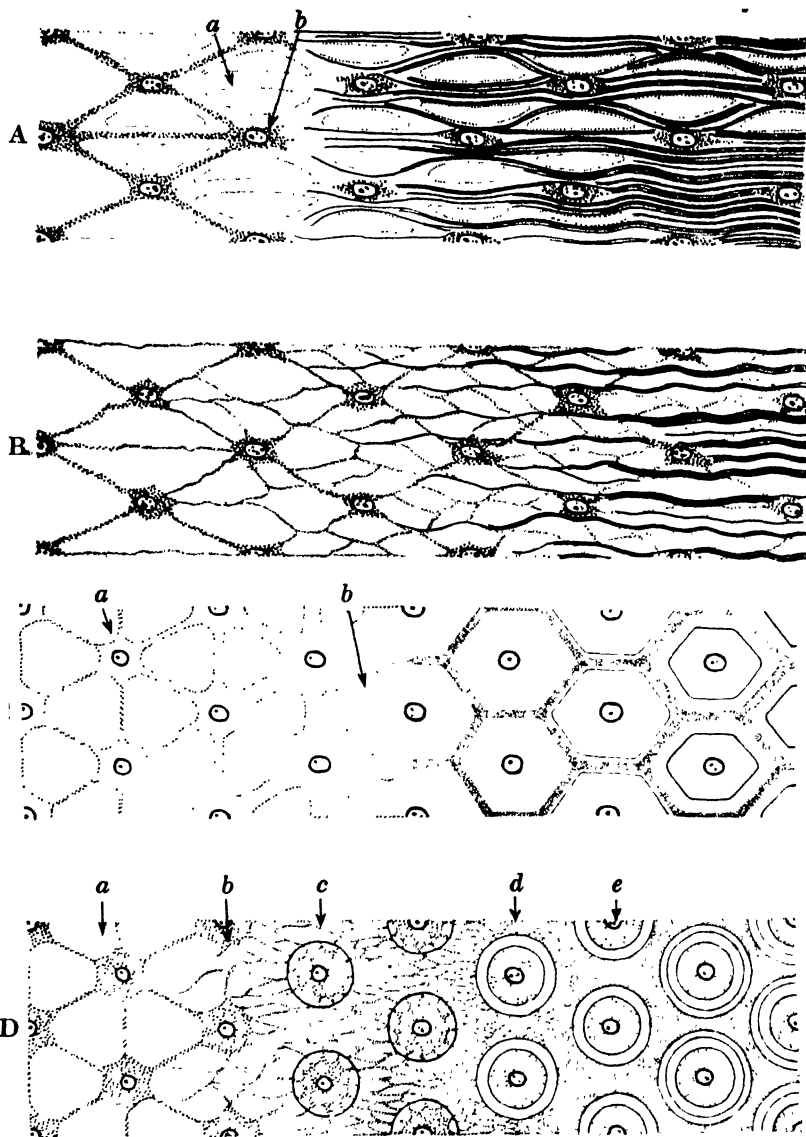


Fig. 4. A and B: diagrams illustrating the development of the loose fibrillar connective tissue of vertebrates. Endoplasm is indicated by large dots, A (left) white, in B marked by fine dots. Connective tissue fibrils are marked by lines. A, my scheme of 1903 (somewhat altered) illustrating the development of exoplasmic layers (lamellae *a*,) with endoplasmic fibrocytes (*b*). (One type of origin of ground substance from mesenchyme.) B, scheme illustrating the participation of the intercellular (exoplasmic) network (secondary mesostroma) in the formation of ground substances and connective tissue fibrils. (Another type of origin of ground substance from mesenchyme.) C and D: diagrams illustrating the development of hyaline cartilage. C, my scheme of 1903 *b* (somewhat altered) showing the development of ground substance from primitive mesenchyme (*a*) on the basis of a plasmodium (*b*). D, scheme illustrating the development of ground substance at the expense of an intercellular (exoplasmic) network (secondary mesostroma). (*a*) Primitive mesenchyme, (*b*) mesenchyme with denser network of cell prolongations, (*c*) young cartilage cells, (*d*) completely differentiated ground substance, (*e*) cartilage cells with their capsules. (After Studnička, 1911 *c*.)

These are sometimes very extensive and complicated. They were known already to Mall (1902), the greater part of his exoplasm splitting into fibres is of this kind. Apart from these "intercellular" protoplasmic networks, a different kind of network exists, viz. the "interdermal network." The latter is represented by the structures described by v. Szily (1904, 1908) (Fig. 5 A). They are formed by cytodemesmata

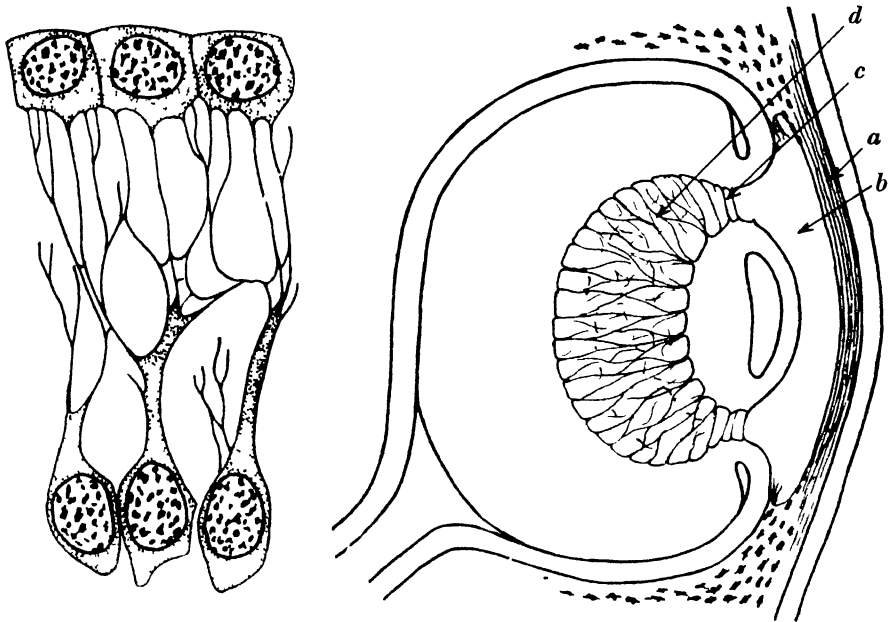


Fig 5 A, origin of the primary (interdermal and exoplasmic) mesostroma between the ectoderm (above) and the mesoderm (below) of a human embryo (after Studnička, 1929) B, diagram of the vertebrate eye indicating the participation of the primary mesostroma network in the formation of the cornea (a), the anterior chamber (b), the zonula ciliaris (c), and the vitreous body (d) (after v. Szily's and Laguesse's statements and my observations on the bird's eye).

connecting two different cell strata, *i.e.* two different germ layers in the embryo, even before the mesenchyme cells enter into these interdermal spaces. The main object of v. Szily's study was the vitreous body of the vertebrate eye which develops between the lens and the retina (Fig. 5 B; see also below, p. 291). I have (later) designated such a network as a "mesostroma" (1911) and have distinguished a "primary" (interdermal) and a "secondary" (intercellular) mesostroma (1911 c, 1912). The origin of the extensive ground substances of cellular or non-cellular gelatinous tissues in particular cannot be found elsewhere than in such exoplasmic "mesostromata" (cf. also Figs. 4 B, D; 10, 11, p. 284).

VIII. GROUND SUBSTANCES OF BONE AND DENTINE.

In 1907 I tried to extend the exoplasm theory to the development of bone and dentine. But before I discuss my own paper I must first deal with v. Korff's papers, published in 1906 and 1907. This author maintained that the ground substance of

dentine and bone has its origin in fibres only, the cells (odontoblasts and osteoblasts respectively) taking only a secondary part in its formation. In his opinion the cells only provide the ground substance by secretions, necessary to its further development. In my paper of 1907 I was not able to share v. Korff's view that at the start there are cells and fibres alone; I pointed out that the fibres were from the start situated in the exoplasm which was transformed into the definite ground substance of the bone or dentine (1) by increase in the number of fibres, and (2) by impregnation with "formative secretions" provided by the osteoblasts or odontoblasts respectively. For my studies (1907 *b*) I did not use the ordinary cellular bone, but the non-cellular osteoid tissue of the teleost fishes. It is evident that the

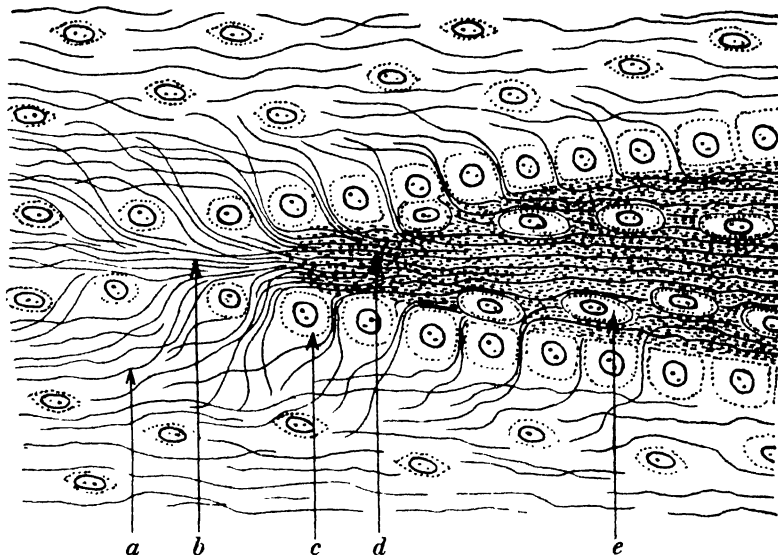


Fig. 6. Diagram illustrating the development of a typical bone on a foundation of embryonic fibrillar tissue (*a*); (*b*) the first commencement of a bone lamella; (*c*) osteoblasts; (*d*) young bone lamella; (*e*) bone cells (osteocytes). (After Hartmann, 1910, and my own observations. The cell connections are not indicated.)

development of ground substance must be essentially the same in either kind of bone; the absence of cells in the osteoid tissue makes the case all the more important¹. In this paper the transformation or exoplasm theory was also extended to the hard non-cellular tissues; their life is a "formative" one.

IX. FURTHER WORK ON THE EXOPLASM THEORY.

The observations of the workers cited above provided all the necessary elements for the construction of an exoplasm theory of the origin of ground substances, or for a theory of the development of ground substance by transformation of proto-

¹ I returned to this theme in 1916. The cellular bone tissue was dealt with by Hartmann in 1910 from the standpoint of the exoplasm theory (Fig. 6).

plasm into exoplasm. (It is necessary to make a distinction between the theory just mentioned and a different "transformation theory" of the origin of *fibres* by transformation of protoplasm.)

Among workers prominent in upholding the exoplasm theory, Laguesse must be mentioned. He called attention (1904) to the especially instructive case of the reticular tissue in the spleen (Fig. 7 A, B) and in the subcutaneous tissue of selachians (1914) and mammals (1921). In 1904 he speaks only of a "precollagenous substance," but later (1914) he also adopted the term "exoplasm." Laguesse called attention to the lamellar structure of subcutaneous connective tissue. The lamellae are formed by exoplasm and the ramified endoplasm cells are situated upon them. The connective tissue fibrils form a part of the lamellae (Fig. 7 C, D, E). In 1926 he proclaimed the mesostroma as a "generally distributed phenomenon," and regarded it also as the basis on which the cornea develops.

In 1913 and 1914 Ranke showed the part played by transformed protoplasm in the histogenesis of mesenchyme derivatives, especially (1914) in the development of the walls of the vessels.

The transformation theory was introduced into the literature of pathology by Hueck (1920). Based mainly on the investigations of other authors, Hueck constructed a very complete "mesenchyme theory."

Studnička (1915) and Florian (1923) used the exoplasm theory also for the interpretation of smooth muscle; its interstitial tissue sometimes exhibits no cells of its own (Florian) and belongs to the exoplasm of the muscle cells (Fig. 7 F).

Lazarenko (1925), Danini (1925) and Zawarzin (1926) have adopted the transformation theory for the interpretation of the genesis of tissues in invertebrates.

Finally, statements (O. Schultze, 1912) concerning the connection between striated muscle and tendon must be mentioned here, according to which myofibrils pass over continuously into collagenous fibrils. In this way a product of the endoplasm (myofibrils) becomes continuous with a product of the exoplasm.

The transformation of ground substances gives an explanation of why all cells in the animal body need not be connected by protoplasmic bridges (cytodesmata). Where the bridges do not exist, ground substance replaces them. Of course, there are also combinations of plasmodesmata with ground substance.

X. OPPONENTS OF THE CELL STATE THEORY: HEIDENHAIN, ROHDE, AND CRITICISM OF THEIR OPINIONS.

Having surveyed the history of the transformation theory¹, we may now return to the critique of the cell theory, or more correctly the "cell state theory." As was stated above, this theory maintains that the body of an animal is "composed of cells." As we have shown, this theory was criticised by the discoverers of "plasmodia" or "syncytia" without any final result. The transformation theory has prepared a new basis for an attack on this problem.

In the first place, I should like to mention Heidenhain who has been prominent

¹ Not all authors who adhere to the transformation theory of ground substances use the term "exoplasm."

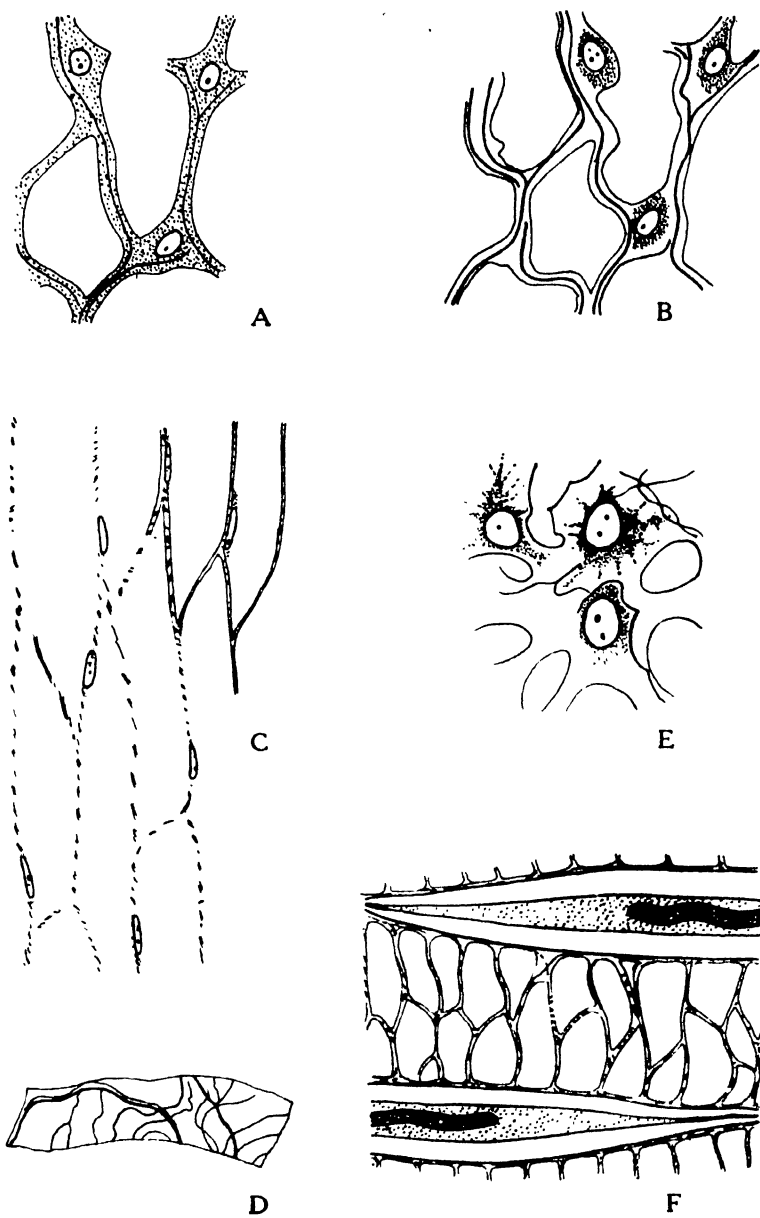


Fig. 7. A and B: reticular connective tissue from the spleen of a selachian: A, an early stage; B, the fully developed tissue with exoplasmic trabeculae (in the sense of Laguesse). C, D, E: lamellar subcutaneous connective tissue of a selachian (*Torpedo*). C, transverse section through the exoplasmic lamellae. D, part of a lamella, containing collagenous fibrils. E, surface view of part of an exoplasmic lamella with endoplasmic fibrocytes (desmocytes) situated upon it. (After Laguesse, 1914; A, somewhat schematised.) F, smooth muscle of the media of an artery of the human umbilical cord. Exoplasmic lamellae (containing numerous fibrils) are situated between the muscle cells. Since the lamellae have the same origin as the latter, the tissue must be regarded as of symplasmic character. (After Florian, 1923, schematised.)

both as an advocate of transformation theories and in the critique of the cell state theory. In 1907 he published a book the title of which, *Plasma und Zelle*, itself indicates his opinion of the significance of the differentiation of the animal body into cells. He supports his views not by his own, but by other authors' investigations, especially those of the supporters of the transformation theory. He adopts their opinion that what I call "formative substances"¹ (cuticular and ground substances) originate by the transformation of protoplasm, and in accordance with the transformation theory he rejects the idea of the cells being the only living structures in the animal body; he accordingly distinguishes a "living mass²," a term which comprises much more than the cells. He differs from most supporters of the transformation theory by the fact that he does not use the terms "endoplasm" and "exoplasm." His "living mass" consists of "protoplasm" and "metaplasm," the latter comprising the fully developed cuticular and ground substances with all their constituents, including the fibres. The vitality of the metaplasm, however, is of a lesser order, only a part of the life phenomena being observable in it. He attributes vitality even to the protomeres (the micellae of Nägeli), but in my opinion we have here to deal only with a "joint life" of the constituents, with a participation in the life of the whole. The main significance of Heidenhain's work is to be seen in the fact that he opposes the official "cell state theory"; he shows its impossibility and he regards the body of an animal as a unit and as an "association of heterogeneous structures" (cells, fibres, bundles, ground substances). But his opposition to the official cell state theory is much better justified than that of Delage and of Labbé, since these two authors did not understand the significance of the ground substances.

Heidenhain has undoubtedly done great service to the acceptance of the transformation theory, but there might be objections to his division of the living substances into protoplasm and metaplasm. Immediately after the publication of his book I suggested (1907 c) that the term "metaplasm" designated not a simple formation (as exoplasm really does) but a complex of the protoplasmic fundament, the fibres and the formative secretions impregnating protoplasm and fibres. In this connection I should like to mention that I (1916) subdivided the ground substances into three kinds: (1) primary, purely exoplasmic ground substances (gelatinous and fibrous connective tissue), where the exoplasm may form loose frameworks; (2) secondary ground substances (cartilage), in which the exoplasm has become impregnated by organic "formative" substances only; and (3) tertiary ground substances (bone, dentine), in which the exoplasm is impregnated with inorganic in addition to organic formative secretions. Further, it might be objected to Heidenhain's terminology that the term "metaplasm" had already been put forward in 1880 by Hanstein for the designation of non-living substances and products of protoplasm which otherwise are called "paraplasm" or "deutoplasm" (the "formative secretions" belong to this category). I fully accepted, of course,

¹ *Bausekrete*, i.e. secretions which penetrate or impregnate the ground substance and make it more resistant; for example, the various organic substances in the ground substance of cartilage.

² The subtitle of his book *Plasma und Zelle* is *Allgemeine Anatomie der lebendigen Masse*.

Heidenhain's opposition to the "cell state theory" and his interpretation of the body of the animal as a unit, since I previously (1902 *b*) interpreted fibres (in particular the fibres of connective tissue, but also other fibres) as elementary constituents of the body of Metazoa, in addition to the cells.

Whilst Heidenhain dealt only briefly in the introduction to his book with data concerning the continuity of protoplasm and the products of its transformation, Rohde attempted in a series of papers to collect all the material relating to this question. In 1908 only cases in which the undifferentiated protoplasm of epithelia, muscles, gonads and other "plasmodia" is continuous were known to him. From his detailed review *Zelle und Gewebe in neuem Licht* (1914) we see that Rohde's attention was called to the origin of ground substances by Heidenhain's book. Here Rohde came to the conclusion that the body of the animal does not represent an "association of cells" but a "plasmodial whole" in which the protoplasm is continuous throughout.

In 1917 Rohde published two short studies on syncytial states during the ontogenesis of animals and in the Protozoa and "Protophyta." Later (1923) he published a new and fully illustrated detailed review of the "plasmodial states" in plants and animals; his conclusions resemble those of Delage and of Labbé. Rohde simply records the observations as he found them in the literature without any detailed analysis¹. The series of cases he mentions is quite convincing, and he showed that protoplasm (in both vertebrates and invertebrates) is almost everywhere actually continuous. He called attention to the fact that true plasmodial conditions exist also in the gonads and give origin to the gonocytes². Biologists seem to have been unpleasantly surprised by Rohde's papers, since the evidence they contain was sufficient to shake the current belief in cells as elementary constituents of the body: they could no longer ignore the evidence that there are many exceptions to schematic "cellular structure."

Rohde draws no distinction between the different ways in which the continuity of protoplasm is effected (he mentions only "plasmodia" or "plasmodial states"), and it can be objected that cases in which cells (epithelium, notochord) are connected by means of numerous "plasmodesmata" differ somewhat from the continuity of the protoplasm in *Muskelkästchen* and striated muscle fibres (Fig. 8); and, again, that the latter are somewhat different from plasmodial tissues (for example the plasmoditrophoblast of the placenta³) or even tissues containing a mesostroma, or, finally, tissues composed of endoplasmic cells and a continuous ground substance of exoplasmic origin. It is necessary to make a more precise distinction between these different conceptions.

Rohde much underestimates the significance of cells and overlooks the fact that syncytial states are very often secondary. It is also necessary to take more into consideration than he did the differentiation of protoplasm into ground substances or fibrous structures. His conception of a plasmodium is too narrow.

¹ See also Schazel's (1918) not always acceptable critique.

² This has recently been confirmed (cf. Ries, 1932).

³ The upper "syncytial" layer of the ectoderm of the trophoblast in man.

In his first paper (1908) Rohde already expressed the opinion that the different cells of the animal body are of different significance. He distinguished primary cells which can be directly derived from blastomeres, and cells which can be formed secondarily in "multinucleated plasmodia" as "free cell formations," or by the disintegration of the plasmodium into cells. In his opinion cells often represent a "very transitory form of the living mass¹." He also used the term "non-cellular tissue" (employed by me in 1907 *b* and actually known to Grönroos in 1903) and gave some examples. He abandoned the current definition of animal tissues which takes into consideration the cells alone. His somewhat popular papers called the attention of biologists far and wide to the conception of the continuity of protoplasm.

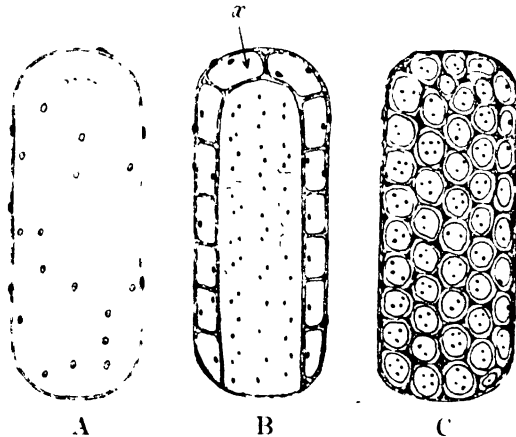


Fig. 8 A, muscle case (*Muskelkästchen*) of the ammocoete: syncytium differentiated into broad lamellae with numerous nuclei. B, muscle case of an adult *Petromyzon*: the prismatic muscle fibres on the periphery (x) separated from each other by connective tissue. C, a complex of cylindrical muscular fibres separated from each other by connective tissue in the corresponding area in *Myxine glutinosa*. (After Maurer, 1894, schematised. All figures represent parts of transverse sections through the myomeres.)

XI. EXTRACELLULAR PROTOPLASM; CRITICISM OF THE ENERGID THEORY; PLASMATOLOGY.

In Rohde's papers only "multinucleated plasmodia" (syncytia of authors) are discussed. I was not satisfied by this and attempted (1913) to distinguish "extracellular protoplasm" from "synexoplasm" ("plasmodium" of Rohde). As I have already (p. 275) mentioned, v. Szily (1904) described extensive protoplasmic networks which developed from the foundation provided by the originally simple "interdermal cytodesmata" (as I designated them later) which are situated between the embryonic layers and the primordia of the organs in an embryo and connect them with one another. In 1907 I described the broad layers of acellular subcutaneous tissue in the pelagic larvae of the teleost fish *Lophius piscatorius*, which

¹ In 1903 I called attention to the significance of what we call "cell" in a somewhat different manner from Rohde. Later (1918) I tried to treat this theme in detail and gave a series of examples.

may be supposed to develop from the above-mentioned networks of v. Szily, becoming transformed later on into an ordinary loose fibrillary and lamellary tissue.

In 1911 *c* I called attention to the fact that such extracellular networks might not only persist and grow between the germinal layers but that they could be secondarily formed, for example, from intercellular cytodesmata of the mesenchyme. I distinguished the "cell bodies" from the "cytodesmic network" or "mesostroma," which, when extensive, are so complicated that they cannot be separated into

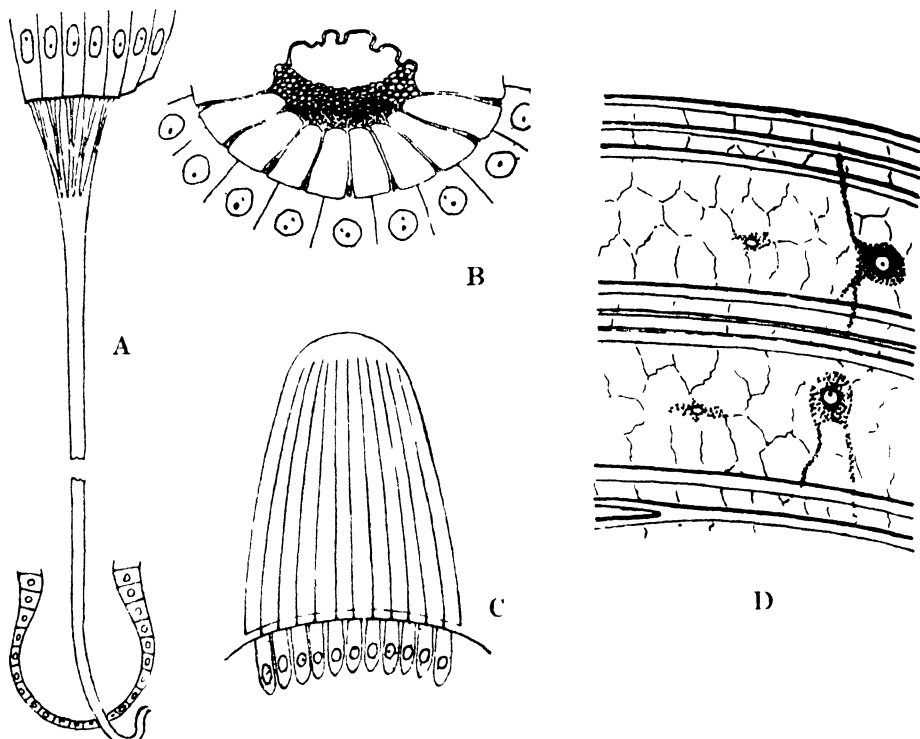


Fig. 9. A, B, C: diagrams illustrating examples of "exostromata." A, Reissner's fibre arising (above) from the ependyma of the subcommissural organ in the diencephalon of a vertebrate and attached to the wall of the ventriculus terminalis of the spinal cord (below); B, primordium of an otolith of a young proammocoetes (after Studnička, 1912 *b*, schematised); C, cupula terminalis on an ampullary crista of a vertebrate. D, extracellular muscles of a platyhelminth worm, connected with the myoblasts only by long plasmatic connections; they originate in the intercellular mesostroma. (After Bettendorff's 1895 statements.)

territories belonging to the cells in question, and are therefore "extracellular¹." In addition, I showed (1912 *b*, 1913) that extensive layers, or structures of a special nature, might originate at the expense of the protoplasm on the surface of cell layers (epithelia) as an "exostroma²" (the opposite of mesostroma). Examples of

¹ There are, of course, also cellular networks in which the protoplasm is clearly under the influence of the cell bodies, as in the subcutaneous chromatophore network of Urodele larvae.

² A complicated network of cell prolongations situated on the surface of an epithelium. The prolongations can occasionally fuse together.

this are, for instance, the otosomata (otolithic membrane, cupula, membrana tectoria in the ear), and Reissner's fibre in the central nervous system (Fig. 9 A). Even the axon and dendrites of the neurons might, in my opinion, be regarded as "extracellular" structures. In this way I was led (1913, 1915) to the idea of "extracellular protoplasm"¹ (Fig. 9 D). It may remain unchanged or it may undergo differentiation into "exoplasm." The latter can form networks or structures some of which may give rise to ground substances. As I showed in 1914, exoplasm can be formed not only on the periphery of cell bodies as autexoplasm, but also extracellular protoplasm can be transformed into a form of exoplasm, for which I have employed the term "synexoplasm." (In 1907 *c* I designated all continuous primordia of ground substances as "synexoplasm.") Recently, I have described the origin of the mesostromatic ground substance of the mammalian amnion (1926), and, together with Florian (1928), in young human embryos of different developmental stages (Fig. 10) (cf. my paper of 1929 *b*). In all these cases there are plasmatic connections (cytodesmata), and, later, networks of cytodesmata (mesostroma) which are partially transformed into a compact gelatinous ground substance (cf. Figs. 4 B, p. 274, 5, 9 D and 11).

In a paper entitled "Conformity and difference in the structure of plants and animals" (1917) I emphasised that attention must be paid not only to the similarity (or conformity) in the structure of both plants and animals, but also to the differences. The differences were made particularly striking by the very recent observations of the formative tissues of the animal body. The plant does not possess an extracellular protoplasm comparable with that of the animals. I called attention to the fact that we must distinguish in addition to a "cell theory," also a "structure theory" and a "protoplasm theory" (*i.e.* a transformation theory). It is evident that to-day the "structure theory" is no longer identical with the "cell theory" in zoology.

Plasmodial or syncytial formations could be explained in the sense of the cell theory as having cells present in them latently as "energids," that is as areas of cytoplasm ruled over by a single nucleus (p. 268)². Extracellular protoplasm does not admit (at least not always) of such an explanation. In addition, we have other cases showing that the cytoplasm need not necessarily be ruled over by special cell nuclei. In 1920 I showed that the lateral trunk muscles of *Amphioxus* represent extensive, practically non-cellular masses of a very active tissue. The myotomes of this animal consist during the embryonic period of cells, but these very soon fuse and a "living mass," not divided into separated structures such as the *Muskelkästchen* of *Petromyzon* or the muscle fibres of craniates, develops; there is no trace at all of a cellular structure. The nuclei of the original cells remain in the periphery of

¹ By "extracellular protoplasm" I understand protoplasm not belonging to the *cell body*, the latter being represented by that part of the cell containing the nucleus. As I have shown (1918, and, more recently, Snessarew, 1932, and Katznelson, 1932), "extracellular" protoplasm may be found not only outside cell bodies but also outside plasmodia. In the latter case, however, the cells are always the original (*i.e.* the first existing) structures. The term "extranuclear" protoplasm is in my opinion incorrect.

² In this sense the syncytia which give rise to the gonads can be explained. In my paper of 1918 I collected a series of such cases and recent literature contains still more of them (cf. p. 280).

this mass and true cells of small size (*Muskelkörperchen*) develop around them and are even capable of division. The rest is actually non-cellular, and no energids or

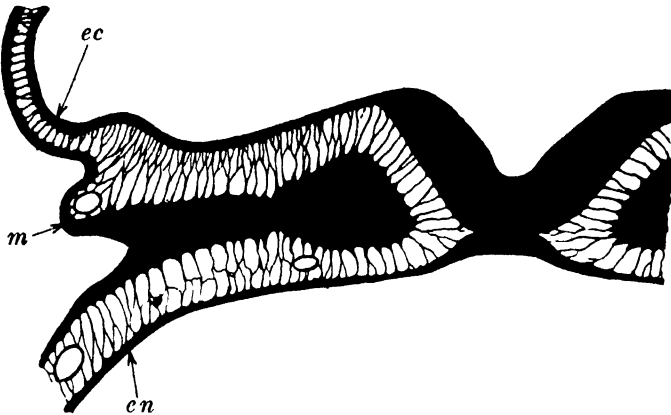


Fig. 10. Transverse section through a young human embryo with 4 to 5 somites. The germ layers are connected with each other by means of cytodesmata and mesostroma *ec*, ectoderm; *m*, mesoderm; *en*, endoderm. (After Studnička, 1929) (Cf Fig 5 A, p 275)

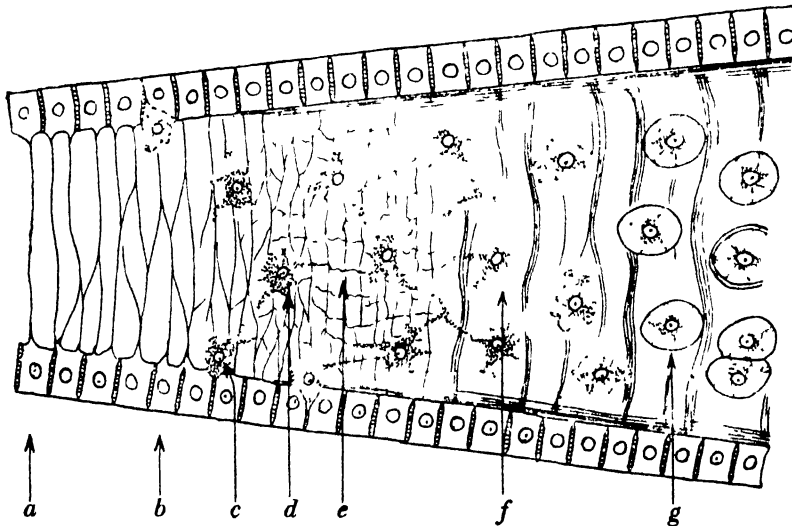


Fig. 11. Diagram illustrating the development of a primary mesostroma (*b*) from interdermal cytodesmes (*a*) with immigration of mesenchymatous elements (*c*), their transformation into cells of mesenchyme tissue (*d*) with an intercellular (secondary) mesostroma, the formation of a gelatinous (pseudo-homogeneous) ground substance (*f*), and the development of vesicular cells (*g*) from indifferent (stellate) mesenchyme cells.

areas ruled over by nuclei can be seen or imagined in it. I am convinced that this very rare case shows that the idea of the essential collaboration of caryoplasm and cytoplasm in the *functions* of the cell must be abandoned. But, on the other hand,

we must not underestimate the great significance of caryoplasm in the transmission of the qualities of tissues.

I took all these circumstances into consideration in describing "The organisation of the living mass" (1929) in v. Möllendorff's *Handbuch der mikroskopischen Anatomie*, and accordingly my treatment of this theme differs to a great extent from that in other histological works. Heidenhain (1907) indicates that "cytology" must not immediately precede "histology," but that a chapter dealing with the different elementary constituents of the animal body ought to be placed between them; he maintains that the conception of protoplasm is broader than that of a cell plasm. I have suggested (1917) the term "plasmatology" as a heading for such a chapter, this term comprising the forms and transformations of the "living mass" in the widest sense of the word. In 1929 I confined myself to the morphological standpoint alone in the elaboration of this problem, and, as themes for such a chapter, I designated, for example, the limiting layers of cells, which have a great significance in the formation of animal tissues, cell connections¹ and their nets, which form considerable parts especially of loose connective tissues; syncytial and plasmodial states of protoplasm, which are important in the formation of muscular tissue; "formative substances" of the body (in the form of ground and cuticular substances), which are completely ignored in cytology; the fibrils and fibres of the animal body, a great part of which is also ignored in cytology; and, finally, the fluids of the animal body (from a quite general standpoint). In my chapter in v. Möllendorff's *Handbuch*, I made an attempt to show how a definition of a tissue, taking into consideration either the cells alone or the cells plus their products, is unnatural. There are (1916) also "non-cellular tissues" composed of living cell-derivates alone, such as "mesostroma" or fibrous formations which must be included in such a definition. We must, so to speak, return to the definition of animal tissue anterior to Schwann's time. In the same *Handbuch* Wassermann (1929) published an extensive chapter describing the "differentiations of the living mass" in which he fully accepts the transformation or exoplasm theory. This is a further proof that conceptions of the structure of the animal body are changing at the present time. Wassermann deals with the question of the genesis of connective tissue fibrils, myofibrils and neurofibrils, which he puts together into one group. In his opinion, which is practically in agreement with my (1902) and Patzelt's (1925) view, they represent differentiations of the living mass.

XII. TERMINOLOGY AND CLASSIFICATION OF SYMPLASMIC STRUCTURES; A NEW DEFINITION OF ANIMAL TISSUE

As stated above (p. 266) De Bary (1859) employed the term "plasmodium" for non-cellular formations which originate by the confluence of cells and thus correspond to a complex of many cells; in 1872 Haeckel designated similar structures (metazoan tissues) as "syncytia." The latter designation clearly indicates that the

¹ Since 1912 I distinguish "cytodesmata" and "plasmodesmata." The cell connections, situated in the cell membranes of plants and similar structures of animals, are plasmodesmata, whilst most of the cell connections of animal tissues (the function of which is to connect the cells firmly with each other) are cytodesmata.

author had in mind a structure corresponding to a complex of undelimited "cells." This circumstance, most probably, was the reason for the general acceptance of this term in subsequent years for all cases of continuity of cytoplasm. "Syncytial epithelia" were distinguished, among which clearly differentiated cells were connected by means of cytodesmata (for example, the stratum spinosum of the epidermis); on the other hand muscle fibres were included in the same group, although they represented protoplasmic cylinders not differentiated into cells. "Syncytia" are defined in this sense even in text-books published quite recently. Bonnet (1903) designated as "syncytia" structures or masses originating by the confluence of cells, and as "plasmodia" structures originating by the division of nuclei not followed by division of the cytoplasm. Bonnet's nomenclature was not

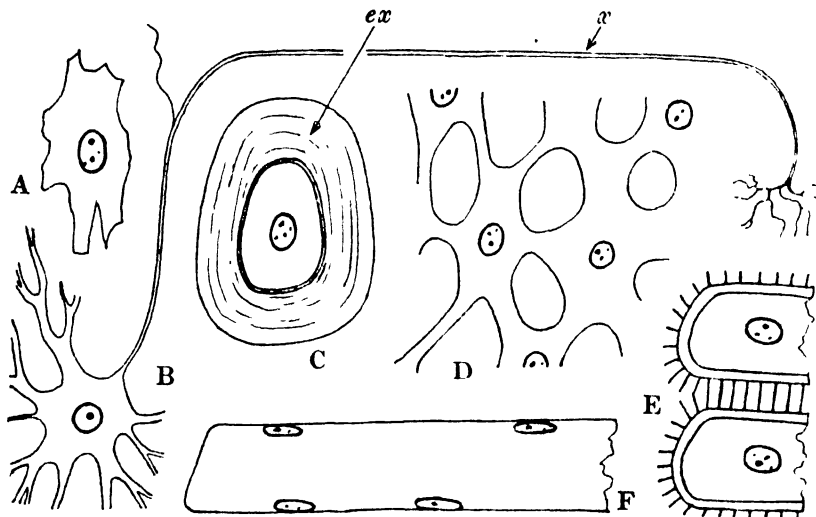


Fig. 12. Different forms of cells and symplasmic states from the animal body. A, cell; B, "cyton" (a cell with prolongations (x), e.g. a neuron); C, "holocyte" (a cell with exoplasm (autexoplasm, ex) belonging to it, e.g. cartilage cell with a broad exoplasmic capsule); D, reticular "syndesmium," cells with cell connections (on the left) passing over into a reticular "plasmodium," or a continuous protoplasmic mass with several cell nuclei (on the right); E, "syndesmium" composed of cells joined by cell connections; F, "syncytium," a well delimited mass of protoplasm with numerous cell nuclei. (Studnička, 1927, 1928.)

generally accepted because it is impossible to determine the mode of origin of a fully developed tissue, and especially because there are cases in which the cells first unite, but later only the nuclei undergo division, while the protoplasm remains undivided. For this reason the term "syncytium" prevailed. I suggested (1903, 1911 b) the term "symplasma" for such structures¹, but later (1928) I preferred De Bary's and Rohde's term "plasmodium" because it was older and more indifferent, and therefore more generally applicable.

I tried to regulate the terminology in 1928 (Fig. 12). I distinguished (1) "syn-

¹ Hanstein (1880) called multinucleated plant cells "symplasts" in contradistinction to uninucleate "protoplasts." Bonnet (1903) understood by "symplasma" a tissue which became non-cellular during degeneration.

desmia" or structures in which the cells, clearly delimited from one another, are connected by means of cytodesmata¹ (epidermis, stratum spinosum); (2) "syncytia" or sharply delimited (often by histological "limiting layers") large structures (*Muskelkästchen* of *Petromyzon* and Annelida, or striated muscle fibres) corresponding to a large number of cells and differing from giant cells by the fact that their cytoplasm is not arranged with respect to the centrioles; (3) "plasmodia" or multinucleate trabecular, laminar or compact *tissues*, formed by confluence of cells or syncytia² or by division of nuclei in growing protoplasm; (4) "extracellular protoplasm" (see above) which can become exoplasmic and be transformed into ground substance with or without a preceding impregnation by formative secretions.

Two objections could be raised against this terminology. (1) I use Haeckel's term "syncytium" in a somewhat different sense. But I find this term so suitable that I do not think it reasonable to abandon it altogether. (2) A series of new terms is suggested. But the structure of animal tissues is so varied that we are obliged to distinguish the main structures of which they are composed by special terms. I regard the tendency in recent literature to employ only the term "syncytium" as very regrettable.

Rubaschkin and Besuglaja have also recently (1932) suggested a new nomenclature. They distinguish protoplasts and symplasts.

The protoplasts represent (1) "cell individuals" which can exist (*a*) as isolated "free cells" as I should call them, or in union with other cells as "tissue cell individuals"; (*b*) as "cell unions" in which the single cells are united with one another to form a protoplasmic whole. In such cases there exists as the cell body a more or less separated protoplasmic territory. (2) Syncytia or cell unions are supposed to represent something different, that is, cases in which the "cell individuality" "has become indistinct through the existence of reciprocal protoplasmic connections, while separated protoplasmic territories have become united to a unitary protoplasmic complex." These structures are identical with my syndesmia.

The symplasts comprise "non-cellular forms," *i.e.* "multinucleate, protoplasmic structures" "in which there is no division of the protoplasmic mass into separated protoplasmic nucleated territories, and accordingly no individualisation in the sense of separation into cell bodies."

As Rubaschkin and Besuglaja (1932) mention, they have borrowed the term "symplasts" from Hanstein, but the latter had (1880) in mind only uninucleate (protoplasts) or multinucleate (symplasts) plant "cells," while the "symplasts" of Rubaschkin and Besuglaja really represent symplasmic animal "*tissues*."

They do not emphasise the differentiation of the protoplasm into exoplasm and endoplasm or protoplasm and metaplasm. They do not take extracellular plasm into account and obviously include it in the "symplasts." I think that this recent classification is no better suited for practical use than that which has been employed

¹ In 1911 I had already maintained that, for example, tissues (epithelium with cytodesmata, mesenchyme) in which the cells are distinguishable (Patzelt has employed the term *Zellverband* for them) must not be designated as "syncytium."

² This was the very reason why I have employed the indifferent term "plasmodium."

by myself for several years. (There is still another classification, suggested by Leontowitsch in 1913, in which cells, syncytia and syncellia are distinguished. For details see the original.)

In connection with this discussion of attempts at classification we must call attention to the fact that the conditions described above are not fixed. Syncytia, or more often plasmodia, may be formed from cells in the metazoan body and these may sometimes become separated into cells again, or new cells may originate endogenously within them. During the segmentation of the egg, cells or plasmodia may develop, as stated above. Meyer (1931) has described a further example of the instability of cellular structure. In *Neochinorhynchus rutili* plasmodia are very soon formed from cells and remain in the subcutis during the whole life of the individual. Monterosso (1926) observed plasmodial germ layers in the copepod *Peroderma cylindricum*; some of the tissues remain in the plasmodial condition throughout life. Lillie (1902), in his classical experiments, suppressed the cleavage of the egg of *Chaetopterus*, and thus succeeded in transforming epithelia into a plasmodial condition.

XIII. EXTREME STANDPOINTS: OPPONENTS OF THE SYMPLASMIC THEORY AND CRITICISM OF THEIR VIEWS.

So far as the continuity of protoplasm is concerned it is now generally accepted that the cytoplasm is continuous in large parts of the metazoan body. It has even become a fashion recently to call attention to such conditions. This tendency is naturally accompanied by exaggerations with which one can hardly agree. Some modern workers have published observations, on tissues previously studied very carefully by other authors, which are very unfavourable to the cell theory, but the observations in question are not above criticism. For instance, Schmidt (1925) describes the epithelium of the primordium of the horse's hoof as a plasmodium in which cells only differentiate later. This tissue was studied earlier by Renaut (1893) and Studnička (1909) and its structure was described as schematically cellular, and quite recently Sajner, in my laboratory, has seen cells very distinctly in surviving tissues. In addition, Schmidt (1930) describes in another paper early stages of the histogenesis of some of the tissues in *Hippocampus* and comes to the conclusion that epithelia as well as muscles grow without any differentiation into cells. I have not examined *Hippocampus* myself, but I have studied numerous differently fixed preparations of another teleost fish (*Lophius*) and have always seen a very distinct cellular structure in the tissues. I presume therefore that this is also the case in *Hippocampus*. It is probable that the fixation of Schmidt's material led the author to his conclusions. I must here mention too Katznelson's (1931) paper on the epidermis of *Salamandrella Keyserlingi*. According to this author the epidermis shows an originally plasmodial condition and the cells only differentiate secondarily. I am convinced that Katznelson was also misled by the faulty preservation of his material and that renewed observations of living specimens¹ will show a typical cellular structure of the epidermis with cells connected by cyto-

¹ In the epidermis of *Siredon* we have observed cells in living specimens (Sajner, 1933).

desmata (as shown by very careful observations of other authors). It is especially the epidermis of vertebrates that exhibits a distinct cellular structure and this is retained very persistently during development (Studnička, 1909).

So much for the epithelia. The conditions in tissues which originate from mesenchyme are quite different. As stated above Sedgwick (1895) showed that mesenchyme is actually a protoplasmic network with nuclei situated in the junctional points of the trabeculae. The view of the "syndesmial" or "plasmodial" (according to my terminology) structure of mesenchyme is now almost universally accepted. But, on the other hand, opinions on the structure of young or fully developed loose connective tissue still differ. Advocates of the exoplasm theory believe that the primary continuity of the protoplasm of mesenchyme persists in its derivatives (loose connective tissue included), that the protoplasm only undergoes differentiation into exoplasm (reticular ground substance) and endoplasm (cells), and that the cells differentiate in several directions, as "tissue cells" and "free cells."

Against this view it is often objected that there is no ground substance at all present in loose connective tissue, and that we have here to do only with cells and connective tissue fibrils, the latter being situated directly in the tissue lymph. But it can be maintained that the ground substance is represented or replaced by quite thin exoplasmic nets not visible in living tissues (as is the case in the vitreous body), which only stain very faintly in preparations and for this reason are very difficult to see. Many authors who adhere to the older views, according to which the ground substance is a secretion of the cells, maintain that the ground substance in some tissues (such as cartilage) is present, while in others (loose connective tissue) it is lacking¹.

Against this objection, it may be mentioned in favour of the transformation theory that cases in which the origin of ground substance by transformation of protoplasm can be distinctly observed are numerous; it is only natural that not all cases are suitable for study. On the other hand there are no observations of a direct formation of ground substances by secretion in most of the derivatives of mesenchyme. I consider this fact as especially important because opponents of the transformation theory overlook it completely and do not realise that the secretion theory is, in most cases, nothing more than a mere supposition. On the contrary, the transformation theory is supported by direct observations, while nobody has directly observed secretions giving origin to ground substances in this first developmental stage. There are, of course, cases in which a secretion actually does pass into the ground substance, but this is always in tissues with a highly differentiated ground substance, such as cartilage, bone and dentine. Here the secretion is always preceded by the formation of ground substance by transformation of protoplasm, and we have to do therefore with an additional and secondary impregnation of a ground substance which existed before the secretion started. Secretions, especially mucin, may be deposited in the clefts of reticular ground substances. Mucin, for instance, is

¹ There are continuous transitional stages between fibrillar connective tissue with indistinct ground substance, mucous connective tissue with abundant ground substance and cartilage in which the existence of a ground substance is undoubted.

present between lamellae of connective tissue in the human umbilical cord. Such secretions do not participate in the structure of the tissue itself, which is based on protoplasm and exoplasm¹. This is the form of transformation theory which I have adopted. It does not, as can be seen, deny the existence of a secretion where such can really be demonstrated. I am convinced that the advocates of the secretion theory, of whom Schaffer is the leading authority, have no clear proofs of their theory.

Among the opponents of the transformation theory I must mention Nageotte. In a series of papers (published since 1905), and in his book *Organisation de la matière* (1920), he expressed the opinion that the intercellular spaces of mesenchyme are originally filled by the *milieu intérieur* of the body. Connective tissue fibrils originate by coagulation of this medium. In his opinion the ground substance is represented by these fibrils only; it is, therefore, a non-living coagulation of a non-living substance. The regular arrangement of the fibres, which is quite evident in numerous tissues, he explains as being due to ferments secreted by the cells. Instead of using direct observations on the tissues in question, Nageotte supports his theory (1927) by observations on the coagulation of fibrin or dissolved collagen. (Collagen coagulates in the form of fibrils which may occasionally form bundles.) In his theory we find no satisfactory explanation of how it happens that the fibrils coagulate sometimes in the *milieu intérieur*, at other times in the fully differentiated ground substance or in the mesostroma as Nageotte himself has described (with Guyon, 1931)². In the latter case, and in the case of extracellular cuticular substances (where fibrils similar to those of connective tissue occur), it is extremely difficult to imagine how remote cells could control the formation of the fibrils.

Another series of objections to the transformation theory was raised by observations on the formation of fibrils in tissue cultures. Maximow (1929) observed the formation of collagen fibrils in tissue culture, not in the cells as one might expect, but between them. Describing this—as he thinks—surprising fact, he was uncertain from which material the fibrils originate. Baitzell (1915) had already expressed the opinion that the fibrils occurring in tissue cultures originate in the medium of the culture, that is, in blood serum of quite a different species, but his opinion was generally rejected. Later Chlopin (1931) suggested that the fibrils originate particularly in the vicinity of the cells (this is not denied by Maximow).

These observations have been used to support the view that the cells do not participate in the formation of fibrils, that is, against the transformation theory. But the authors have forgotten the existence of extracellular protoplasm. Chlopin (1931) has actually seen fine, but distinct, exoplasmic threads which could be traced for some distance from the cell body and might represent, in my opinion, a part of the extracellular protoplasm. Recently Levi-Momigliano (1932) described extremely fine protoplasmic threads in tissue cultures seen with a dark background. The networks of extracellular protoplasm may be entirely transparent and invisible under

¹ Mucin is not a formative substance, but probably a product of the protoplasm and exoplasm.

² Nageotte and Guyon (1931) suppose that in some cases the proteins of the *milieu intérieur* coagulate, in other cases it is the proteins contained in the mesostroma.

normal circumstances, as in the case of the vitreous body (see below)¹. It is important to remember that in other cases the formation of fibrils in cell plasma or directly in compact (*i.e.* non-fibrillar) exoplasm has been undoubtedly demonstrated. (Also in mesenchyme, by Lewis, 1917.) I do not imagine that the formation of fibrils occurs in one case in the living substance (protoplasm), in another case in a secretion, that is, in non-living matter. To close this discussion, I would mention that Friedheim (1930) has described the formation of a real ground substance by transformation of protoplasm in tissue culture.

XIV. EXAMPLES OF NON-CELLULAR STRUCTURES AND TISSUES.

I should like here to mention five examples of tissues, the genesis and structure of which can be easily explained by the transformation theory, but which are inexplicable by the classical secretion and cell state theory.

(1) The vitreous body of the vertebrate eye was originally supposed to be a transudation from the adjacent blood vessels, but later fibrils were found in it and it was observed that they were continuous with the surrounding tissues during embryonic life. Tornatola (1897) supposed that the vitreous body was formed by the embryonic retina; Lenhossék (1903) derived it from the lens; but finally v. Szily (whose work, 1904, has already been mentioned above) observed that the first primordium of the vitreous body was represented by simple cytodesmata connecting the cells of the primordium of the retina with the cells of the primordium of the lens, which, during further development, became much longer and more complicated, forming a dense network (*cf.* Fig. 5 B, p. 275). The network is afterwards transformed into fibrils. But later on doubts were raised about the real existence of these structures visible only in fixed preparations, and still more recently (1932) Redslob has expressed the opinion that the vitreous body is nothing but a homogeneous sol which coagulates into fibrils under the influence of the fixative. But Baurmann and Thiessen succeeded (1922) in seeing these fibrils distinctly in living eyes by means of the *Spalt-Immersion-Ultramikroskop* of Zsigmondy. They really are the same fibrils (Heesch, 1927) which have been described in fixed preparations and there is evidently no reason to deny their existence. Recently (1933*a*) I have succeeded in making these fibrils distinctly visible in fresh surviving objects by means of Indian ink or collodal silver. The vitreous body, accordingly, represents a non-cellular tissue, formed by transformation of protoplasm into a kind of exoplasm and originating in a primary mesostroma.

(2) The zonula ciliaris of the eye of vertebrates originates from the same primordium as the vitreous body, that is, from a protoplasmic network (mesostroma) situated between the lens and the corpus ciliare. The fibres (which remain thin in the vitreous body) here become very thick (up to 100 μ in diameter in some mammals). The complex of these fibres represents a kind of non-cellular fibrous tissue (zonula ciliaris) attaching the lens to the corpus ciliare. Cellular histology has no acceptable explanation for such a structure.

¹ Recent observations of mine (1933*c*) made after having written the present article prove the exactitude of my suppositions.

(3) The enamel of the dentine teeth of vertebrates was originally considered to be a secretion of the ameloblasts. Later it was supposed that it originated from the prolongations of ameloblasts which calcified into the enamel rods. Recent observations show that the enamel originates as an independent layer between the ameloblasts and the young primordium of the dentine. (Williams in 1895 already expressed a similar opinion.) I was able to show (1917 *b*) that an extracellular protoplasmic network was formed by the connections of the odontoblasts and ameloblasts and that the calcium salts were deposited in special sac-like structures from which the enamel prisms develop. The enamel represents, therefore, an extracellular, non-cellular tissue.

(4) The otosomes of the organ of hearing of vertebrates, by which term I designate all specific auxiliary structures situated upon the sensory epithelium which enable the organ of hearing and the static organ to function, *i.e.* the otoconial membrane, the otolithic membrane (as the support of the otoliths in the Ichthyopsida), the cupula terminalis and the tectorial membrane of the organ of Corti. These structures have been regarded as secretions and it was even suggested that the cupula was an artificial product. (But the latter has already been seen *in vivo*. Steinhausen (1927) proved its existence by means of methylene blue and Indian ink.) All these structures originated from a foundation of extra-cellular protoplasm derived from the sensory cells (1912 *b*). Wittmaak (1918) and his school have recently expressed the opinion that they represent active parts of the organ of hearing (cf. also Werner, 1932¹). Here, too, we have to do with transformed protoplasm (exoplasm). (Fig. 9 B, C, p. 282.)

(5) Reissner's fibre of the canalis centralis of the central nervous system of vertebrates is constituted by a strand of relatively firm substance which can be traced from the so-called "subcommissural organ" below the commissura posterior through the mesencephalon and myelencephalon to the caudal end of the canalis centralis of the spinal cord. Here it is attached to the surrounding connective tissue outside of the sinus terminalis. After rupture it can regenerate and its fixation to the end of the spinal cord can be restored. It must not therefore be regarded as a non-living structure but as a strand of stiff extracellular protoplasm which originates from the subcommissural ependyma. Kolmer (1921) has designated the organ, the most important part of which is represented by Reissner's fibre, as the "sagittal organ." (Fig. 9 A, p. 282.)

I have mentioned here only these five examples of extracellular tissues or structures, which, because they contain no cells, were designated not as "tissues" but as "masses" by the older histologists. It is evident that the definition of a "tissue" must be altered so as to include such structures. The tissues of the metazoan body may be composed of (or contain) not only cells but also syncytia or plasmodia, or they may be altogether non-cellular. Fibrils may form constituents of a tissue in the same manner as cells (cf. p. 280).

It is not correct to speak of a "plasmodial state" of the whole organism as Rohde does; the plasmodia, syncytia and the extracellular protoplasm must be

¹ Werner now calls them "sensularia." The term "otosome" is older and more accurate.

regarded as structures in a certain sense equivalent to the cells and as having a similar significance as components of the tissues. But we are entitled to speak of a "symplasmic condition" of the organism¹, if we have in mind the fundamental continuity of its original or differentiated protoplasm.

I have shown in this article that cellular histology merges into "non-cellular." Even the latter, of course, considers cells as the original, and, in typical cases, the main constituents of the animal body, but takes into consideration also the syncytia, plasmodia, networks, masses, ground substances, fibrils and fibres; in accordance with Heidenhain, it considers the animal body as a complex of very different constituents.

XV. TERMINOLOGY OF BIOPLASMS.

It will now be appropriate to reconsider the principal terms applicable to the living substance which have hitherto been in use. It is evident that the very considerable changes in our point of view will necessitate alterations in the terminology. I would here call attention principally to the terms "cytoplasm" and "protoplasm." The former was first employed by Kölliker (1867) for the same extranuclear substance of the cell for which Mohl (1846) used Purkinje's term "protoplasm." Strassburger (1875) included under the term "protoplasm" also the living substance of the nucleus, for which he introduced the term "nucleoplasm" in contradistinction to "cytoplasm," and Flemming (1882) changed this term into "karyoplasm." Later Haeckel (1873) made a further division of protoplasm into "endoplasm" and "exoplasm." Only the protoplasm of the cell body was taken into consideration, and exoplasm represented the rind substance of the cell.

But the discovery of "extracellular" protoplasm (present in the form of prolongations, cytodesmata, networks, masses and layers, and contained in ground and cuticular substances) has necessitated a more indifferent designation than the term "cytoplasm," free from any reference to the "cell."

Objection might be raised even to the term "protoplasm" itself. Literally translated it designates that which was "created first" and it was originally used (Purkinje, 1839) in this very sense, that is, to mean the substance of what we now call the cells of the embryonic body. Mohl (1846) employed it for what we call cytoplasm, but Strassburger (1875) and Flemming (1882) meant by this term the whole living substance, that is, what Huxley (1868) called "the physical basis of life." But there are great differences in the condition of the substance which can be so designated, and it is not possible to suggest that all such conditions are "proto"-plasm in the literal sense. The basis of myofibrils, ground or cuticular substance, even if it be living, is not "protoplasm"; if we call it "metaplasm," in accordance with Heidenhain, we arrive at other incongruities, as stated above (p. 279). It is not possible to designate as "protoplasm" substances which differ too much from the original living substance, just as it was not possible to use the term "cytoplasm" for extra-cellular substances; the latter term can be used only for the cell body, not where the whole tissues are concerned.

¹ Bertalanffy (1933) recently employed the term "symplasma" in a sense similar to my term "symplasmic state."

In 1918¹ I subdivided the "basis of life" (of which there evidently exist different kinds) into the following substances: (1) "proplasm" or plasma of the resting nuclei; (2) "protoplasm" or "endoplasm," that is, the most primitive formed plasma, usually surrounding the nuclei and containing all organoids of the cells, forming the bodies and prolongations of the cells, and sometimes also situated among or outside the cells; (3) "exoplasm" on the surface of the cells, or present among or outside them; (4) "paraplasm," present in functional structures (fibres), situated in both protoplasm and exoplasm; (5) "rheoplasm," forming the fluids which participate in the living processes of the organism.

According to this terminology "protoplasm" and the "physical basis of life" would not be identical, and it would be necessary to find a term for the whole complex of living substances. Heidenhain (1907) once introduced for his "protoplasm plus metaplasm" the term "living mass," and recently v. Möllendorff has made use of it in the title of the first volume of his *Handbuch der mikroskopische Anatomie* (1929). But there exists an international scientific term, "bioplasm," employed (1876) by the English histologist and physician Beale for the designation of the living mass, to which I called attention in 1918. This term seems to me to be a very suitable one.

XVI. SUMMARY.

The metazoan body is not "composed of cells," as is usually asserted, but, besides the cells, it contains other protoplasmic structures and masses. These are (1) *syncytia* (for example a muscle fibre); (2) *plasmodia* (whole tissues formed of protoplasm, but showing no differentiation into cells); (3) *intercellular* networks formed by *cytodesmata*, situated between cells or syncytia; (4) extensive *extra-cellular cell prolongations* (that is prolongations situated outside the cell bodies, for example in the nervous system).

The so-called *ground substances* are structures which originate not by secretion, but by the transformation of protoplasm. (Only mucous layers situated between the structures of the body originate by secretion.) *Mesostromata* (fine networks of cell connections) are the principal material from which ground substances originate. They may consist of modified protoplasm or *synexoplasm*; *autexoplasm*, or modified protoplasm (*exoplasm*) on the periphery of cells (for example in cartilage) represents another material from which ground substances may originate.

In such tissues the structures which are designated as *cells* by authors really represent the *endoplasms*, whilst the exoplasm (or ground substance) situated between them is not divided into cell territories and, accordingly, constitutes a *symplassmic* structure.

Fibrillar material, for example precollagenous and collagenous fibrils and elastic fibres present in a connective tissue during its development, arises in both the autexoplasms (which may join together) and the synexoplasms (which may exhibit varying reticular or lamellar forms). We can distinguish (1) ground substances consisting of pure exoplasm (containing fibrils only); (2) ground substances

¹ In 1911 I suggested the term "somatoplasm" instead of "cytoplasm," but it has previously been used in quite a different sense, and, in addition, is not sufficiently expressive.

composed of exoplasm impregnated by organic "formative secretions" (products of the cell bodies) only; and (3), as is the case in bone and dentine, impregnated first by organic secretions and subsequently by inorganic salts in addition.

Cuticular substances are analogous to ground substances and possess the same potentialities.

Ground substances consist of living matter, at least during their development, and we must therefore not regard cells as the only living components of the metazoan body nor designate the latter as a "cell state." Fibrils, for example, are components of the fully differentiated body which are as important as cells (and syncytia). The current definition of an animal tissue should be replaced by one comprising all living structures and substances since "non-cellular" exist as well as "cellular" tissues. "Protoplasm" is not the only existing form of living matter; there are different phases of *bioplasm*, that is, of Heidenhain's "living mass," in modern "non-cellular" histology. A metazoan body is, accordingly, in a *symplasmic* state in which the living substance is continuous throughout.

In conclusion, I desire to express my thanks to Prof. Florian for his kindness in translating the present article into English, and to Prof. Munro Fox for revising it.

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¹ For a full account and complete bibliography of the subject up to 1929 the reader is referred to my chapter, "Die Organisation der lebendigen Masse," in v. Möllendorff's *Handbuch der mikr. Anat.* **1**.

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THE ENDOCRINE GLANDS AND CALCIUM METABOLISM

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CONTENTS.

	PAGE
I. The thyroid	300
(1) Excretion of calcium	300
(2) The serum calcium level	304
II. The gonads	306
(1) The effect of gonadectomy on serum calcium	306
(2) Injection of gonadal extracts	307
(3) Serum calcium in menstruation and pregnancy	308
(4) Serum calcium in relation to the ovarian cycle in birds and Amphibia	310
(5) Sex differences in the calcium content of the serum	312
III. The pituitary	313
IV. The adrenals	316
(1) Effect of adrenaline on serum calcium	316
(2) Adrenalectomy	318
(3) Injection of cortical extracts	319
V. The pancreas	319
(1) Serum calcium in diabetes and after injection of insulin	319
(2) Pancreatectomy	321
VI. The lymphocytogenic organs	321
(1) The thymus	321
(a) Thymectomy	321
(b) Injection of extracts	322
(c) Relation of the thymus to the secretion of egg envelopes in birds	325
(d) The thymus and the calcareous bodies of Schwammerdam	326
(2) The spleen	326
VII. The pineal	327
VIII. Summary	327
References	328

A NUMBER of reviews on several aspects of calcium metabolism has appeared recently: "Calcium metabolism," Stewart and Percival (1928); "Calcium and phosphorus metabolism," Aub (1928), Hunter (1930); "The parathyroid glands," Thomson and Collip (1932); *Calcium Metabolism and Calcium Therapy*, Cantarow (1933). In almost all of these the dominant rôle played by the parathyroid glands has been emphasised. In recent years, a considerable amount of data has accumulated which indicated that some of the other glands of internal secretion may play

a part in the regulation of calcium metabolism. In the following review an attempt has been made to collect and evaluate this evidence.

Although the lymphocytogenic organs (thymus, spleen and lymph glands) do not strictly come under the heading of endocrine glands, the results are sufficiently interesting to be included.

I. THE THYROID.

(1) EXCRETION OF CALCIUM.

Our knowledge in regard to the relationship between the thyroid gland and calcium metabolism was put on a sound basis by the work of Aub and his collaborators. In the first communication on the subject (Aub, Bauer, Heath and Ropes, 1929) a short summary of previous literature is given. As early as 1892 Koeppen reported that there appeared to be a connection between exophthalmic goitre and osteomalacia and other bone diseases. Scholz (1905) reported the results of studies of the metabolism of cretins before and after thyroid therapy. The untreated cretin retained phosphorus and excreted large quantities of alkaline earths. Administration of thyroid preparations exerted no marked influence on phosphorus metabolism but reduced the excretion of alkaline earths in the urine, especially that of calcium. The faecal calcium, however, was increased. Other results reported about the same time were of less value as they were based on unsatisfactory methods and incorrect assumptions, or were due to insufficient dosage with thyroid and too short a period of observation. An important contribution to the problem was made by Parhon (1912). The calcium exchange of nine rabbits was determined both before and after the administration of thyroid. One group of animals received 0.05 gm. of thyroid daily and lost 0.007 gm. calcium per kg. per week. A second group of three, all of which died within 17 days, received 0.10 gm. thyroid daily and lost 0.228 gm. (average figures) per week; the last three died within 5 days after the beginning of thyroid administration. They received 0.3 gm. thyroid daily and lost, on the average, 0.662 gm. calcium per kg. per week. Thus administration of thyroid caused a negative calcium balance, and the loss of calcium was roughly proportional to the dose of thyroid. These results indicated that thyroid substance stimulates the excretion of calcium. Kojima (1917) removed the thyroid and parathyroids from rats and then replaced the latter. Calcium excretion was diminished but administration of thyroid did not affect calcium metabolism. No data are given in regard to the adequate functioning of the parathyroid transplants, and the reduced calcium excretion after thyroidectomy might have been due to parathyroid deficiency. Kummer (1917) reported as the result of a 13-day observation of a patient with exophthalmic goitre who was given $2\frac{1}{2}$ litres of milk daily, *i.e.* about 5 gm. calcium daily, that the mineral excretion was high but very irregular, both calcium and phosphorus losses being especially large. The quantity of calcium in the urine was normal and that in the faeces was large, and he concluded that the high faecal calcium was due to a difficulty in absorption rather than to abnormal secretion.

Deuel, Sandiford, Sandiford and Boothby (1928) found that the injection of 7 mg. of thyroxine into a subject who had been maintained on a prolonged protein-

free diet had no effect on the excretion of urinary calcium. This negative result is probably due to the small dosage and to the fact that the diet was not specially planned for an investigation of calcium metabolism. Aub, Bauer, Heath and Ropes (1929) studied the calcium, nitrogen and phosphorus excretions in a series of patients with various thyroid diseases. In a previous paper Bauer, Albright and Aub (1929) pointed out that determinations of serum calcium alone give no information in regard to whether calcium is passing into the excretory channels or into the bones. Serum calcium estimations must be supplemented by analyses of calcium intake and output. The faecal calcium represents calcium which has passed through the intestines unabsorbed and calcium which has been absorbed and re-excreted. Only the latter can be regarded as actively taking part in calcium metabolism. In studies on calcium metabolism, therefore, the calcium intake should be as low as possible, the diet being adequate in other respects. Certain minimal quantities of calcium are necessary to keep the body in calcium balance. The negative calcium balance on a zero intake of calcium might be regarded as an index of endogenous calcium metabolism. The low calcium diet not only permits of a more accurate evaluation of endogenous calcium metabolism but also largely eliminates the unknown factor of unabsorbed calcium found in the faeces. In the studies on the effect of thyroid disease both normal individuals, thirteen in number, and patients were given a daily diet with a very low calcium content but otherwise adequate. It was found that the calcium excretion in patients with exophthalmic goitre or in those with hyperfunctioning thyroid adenomata was markedly increased as compared with the calcium excretion of the normal standards. When calculated for 3-day periods the average excretion in the normal individual is 12.7 mg. per kg. body weight, and in patients with exophthalmic goitre 42.0 mg. per kg., an increase of 231 per cent. above normal. In one case 4.6 gm. of calcium were excreted in 3 days instead of 0.79 gm., the average for the normal individual. Administration of thyroid extract plus thyroxine to two normal individuals increased the excretion of calcium. In six cases of myxoedema the average excretion of calcium was 40 per cent. below the normal average. Thyroid feeding led to an increase in metabolic rate and in the excretion of calcium. By means of X-ray examination the presence of osteoporosis was established in a patient who was known to have had exophthalmic goitre for 17 years. This observation was confirmed by Plummer and Dunlap (1928). Stettner (1931) described a child aged $5\frac{1}{2}$ years who had been given 0.05 gm. thyroid per day for 3 years from the age of two. This patient showed marked osteoporosis in the bones of the hand and legs. The increase in phosphorus excretion in patients with exophthalmic goitre was quantitatively such as to suggest that most of the calcium excreted came from tertiary calcium phosphate in the bones. Some of this calcium, however, may, as suggested by Goto (1918), represent calcium carbonate withdrawn from bones.

The increased calcium excretion in hyperthyroidism may be due to an indirect effect of an increase in metabolic rate as indicated by Percival and Stewart (1926). Aub, Bauer, Heath and Ropes, however, present evidence which suggests that this is not the correct explanation. In other cases of increased basal metabolism, two

patients with prolonged fever and one with leucaemia, the calcium excretion on the routine diet was normal. A fourth case, suffering from myelogenous leucaemia, showed an increased calcium excretion approaching that found in hyperthyroidism. Neutralisation of the acid products of increased metabolism might account for the increased excretion of calcium. Studies by Albright, Bauer and Aub (1931) on the total acid-base metabolism in hyperthyroidism and myxoedema indicate that the thyroid hormone does not bring about mobilisation of calcium phosphate to assist in the excretion of acid metabolites. The effect of thyroid on calcium excretion may be due to an associated hyperparathyroidism, but the following facts do not support this suggestion. Albright, Bauer, Ropes and Aub (1929) point out that in hyperthyroidism the serum calcium is only slightly increased and there is an increased calcium excretion in both faeces and urine, while in hyperparathyroidism the serum calcium is markedly increased and the increased calcium excretion is entirely urinary, the faecal excretion being unaffected. An increased calcium output in both urine and faeces is found only in hyperthyroidism and vitamin D deficiencies. The possibility that hyperthyroidism may be associated with vitamin D deficiency because of the increased metabolic demand and an increased need for vitamins was investigated by Tibbetts, McLean and Aub (1932). Large amounts of irradiated ergosterol were given to hyperthyroid patients maintained on the routine diet, but no decrease in the excretion of urinary or faecal calcium was observed. Cowgill, Gilligan and Blumgart (1930) reported a case of osteomalacia with high urinary and faecal calcium which returned to normal after large doses of vitamin D. Bauer and Marble (1932) found that irradiated ergosterol caused a precipitous fall in the faecal calcium of a patient suffering from steatorrhoea. The excretion of calcium in the faeces fell from 3.74 gm. in the control period to 0.16 gm. during ergosterol therapy.

The most likely explanation for the increased excretion of calcium in hyperthyroidism is, as suggested by Aub and his collaborators, a direct stimulating effect on the calcium deposits in the bones. That a readily available reserve supply of calcium does exist in the bones was suggested by the work of Hunter and Aub (1926), and that this labile depot for calcium is located in the bone trabeculae was demonstrated by Bauer, Aub and Albright (1929). Parhon, Derevici and Derevici (1932) treated dogs with powdered thyroid (0.2–0.3 gm. per kg.) or with 1 c.c. thyroxine a day (8–23 days treatment for thyroid and 5–8 days for thyroxine). In both cases a definite decrease in the calcium content of bone occurred.

These conclusions in regard to the important part played by the thyroid in calcium metabolism are reinforced by investigations showing the effect of thyroid administration in cases of hypoparathyroidism. Kunde, Oslund and Kern (1931) found that in 55 per cent. of a series of fifty-one dogs the tetany following thyroid-parathyroidectomy was temporarily controlled by experimental hyperthyroidism. Desiccated thyroid per os or thyroxine intravenously maintained the serum calcium above the tetany level for 1–3 weeks. A decrease in the serum calcium followed the discontinuance of thyroid administration. They concluded that the thyroid is closely associated with the mobilisation and excretion of calcium. Aub, Albright, Bauer and Rossmeisl (1932) reported the effects of thyroid administration in two

cases of parathyroid tetany. One case received daily intramuscular injections of 15 units of parathormone. This raised the blood calcium from 4.2 to 6.7 mg. per 100 c.c. during a period of 18 days. Without altering the parathormone dosage, thyroxine (25.0 mg.) and thyroid (2.4 gm.) were administered during a period of 2 weeks. The metabolic rate rose from -14 to +22 per cent. and the blood calcium from 6.7 to 11.7 mg. It was only during this period that the excretion of calcium was influenced, increasing threefold over its value in the earlier periods. The increased calcium in the blood and excreta persisted for 6 weeks after thyroid administration was discontinued. There was also an increase in urinary phosphorus which began almost immediately. The serum phosphorus was little affected. In the other case parathormone alone raised the serum calcium from 6 to 7.8 mg. without much effect on the urinary calcium. Thyroxine was then injected intramuscularly and the serum calcium rose to 9.4 mg. The urinary calcium was also increased. The authors point out that, whereas in hyperthyroidism, in spite of a marked increase in calcium excretion, there is no definite increase in blood calcium, in hyperparathyroidism administration of thyroid or thyroxine results in considerable rise in blood calcium. This rise does not lead to an increased excretion of calcium in the urine until the blood calcium has risen beyond the threshold level. Albright and Ellsworth (1929) found in a case of idiopathic hypoparathyroidism that as the serum calcium rose from 5.2 to 11.2 mg. per 100 c.c. following the injection of parathormone, there was a critical serum value of about 8.5, at which point an almost negligible urinary calcium excretion suddenly changed to a very appreciable one. When the serum calcium was above 8.5 the urinary calcium increased as the serum calcium rose, then decreased abruptly as soon as the serum calcium fell below 8.5 mg. per 100 c.c. This suggests that there is a threshold for urinary calcium. Aub, Albright, Bauer and Rossmesl (1932) comment as follows on these results: "The extraordinary thing about this threshold is that it is surpassed by the normal value for serum calcium. Calcium privation, unless long continued, will not lower the serum calcium to the threshold value. There is no counterpart to this in physiology as far as we are aware. The implication is that there is another mechanism which keeps the serum calcium above this threshold otherwise the calcium excretion in the urine would soon lower the serum calcium to the threshold value. The other mechanism may well be the parathyroid hormone." To explain the fact that thyroid administration has this striking effect on the serum calcium of hypoparathyroid patients while the increase in normal patients is slight, they advance the following hypothesis: the thyroid hormone mobilises large amounts of calcium from the bones into the blood stream and thence into the excretory channels. A slight rise in the blood level of calcium occurs, and because the threshold is then exceeded the calcium is immediately excreted. In hypoparathyroidism the calcium on arriving in the blood stream finds itself in the blood below rather than above the threshold for excretion and hence is not immediately excreted. The serum calcium therefore rises.

(2) THE SERUM CALCIUM LEVEL.

Changes in the calcium content of the serum in thyroid diseases and following thyroidectomy or thyroid administration have been extensively investigated. Leicher (1922), whose results have been widely quoted, found that administration of thyroiodine tablets to human beings for 3-4 weeks led in five of six cases to a diminution in serum calcium of 4-11 per cent. Three cases of exophthalmic goitre showed a serum calcium level about 10 per cent. below normal, and one case of myxoedema an increase of about 10 per cent. The differences found fall within the normal variations, and the number of cases is too few to warrant any definite conclusion. Normal values in hyperthyroidism were reported by Kneschke (1923). Jansen (1924) found low values in several cases of hyperthyroidism. Herzfeld and Neuberger (1924) examined nineteen cases of hyperthyroidism and found normal values in about one-third of the cases, in the rest the calcium level was either above or below normal. Castex and Schteingart (1925) found no change in hypo- and hyperthyroidism. Waldorp and Trelles (1926) investigated twenty-six cases of thyroid disease with increased basal metabolism, but in only four of these was the blood calcium reduced. No direct relationship between the basal metabolism and the level of blood calcium could be established. Percival and Stewart (1926) reported a normal serum calcium level in a case of cretinism. Montanari (1928) found the serum calcium level increased in eight cases of hyperthyroidism and decreased in one case of hypothyroidism. McCullagh (1928) found the serum calcium to be normal in 94 per cent. of 139 cases of hypo- and hyperparathyroidism. Wade (1929) reported a serum calcium of 8.3 mg. per 100 c.c. in eleven patients—exophthalmic goitres or toxic adenomas of the thyroid. The normal level determined by the average of ten normal individuals was 10 mg. The serum calcium was consistently lower in the untreated cases before operation. The more toxic the case the lower was the calcium reading. After operation all the cases showed an increase in serum calcium to 10.8 mg. (average). Aub, Bauer, Heath and Ropes (1929), in their carefully controlled investigation, found no marked variation from the normal in most of the fourteen cases of thyroid disease studied by them. In hyperthyroidism there was a slight and almost negligible increase in serum calcium. Ask-Upmark (1932) found normal values in several cases of hyperthyroidism.

An increase in serum calcium after thyroid administration was reported by Oltramare (1924), by Rothschild and Jacobsohn (1927) and Nishimura (1928) in man; by Trifon (1929) and Blinoff (1930) in guinea-pigs, and by Horsters (1930). The last-named observer injected 1 c.c. of thyroxine daily for 3 days into rabbits (two animals only) and obtained increases of 21 and 27 per cent. Aub, Bauer, Heath and Ropes (1929) administered thyroid and thyroxine to two normal individuals. There was an increased excretion of calcium but the serum calcium remained normal. Kunde, Oslund and Kern (1931) found that experimentally induced hyperthyroidism (desiccated thyroid per os or thyroxine intravenously) increased the level of acid-soluble phosphorus but did not affect the serum calcium in dogs. They conclude that in hyperthyroidism the mechanism for the maintenance

of the normal calcium level in blood is unimpaired. The suggestion that the effects of hypo- or hyperthyroidism might be due to changes in blood volume was investigated by Kunde, Green and Burns (1932). They showed, however, that in these conditions the total blood volume of rabbits does not change beyond the normal variations. The bulk of the evidence reviewed above indicates that serum calcium is subject to slight variations in thyroid disease and after thyroid administration. That there is a tendency to slightly increased values in hyperthyroidism seems to be fairly well established.

The evidence in regard to the effect of thyroidectomy on serum calcium is contradictory. Hug (1921) reported that thyroidectomy had no effect on the calcium content of the serum in cattle. Parhon (1923) thyroidectomised three sheep at the age of 6 weeks, and 1 year after the operation found a decrease in the calcium content of whole blood as compared with controls of the same age. Leites (1924) extirpated the thyroid in dogs. In one animal the serum calcium began to rise from the 5th day after the operation and after 15 days had increased 25 per cent., at which level it remained. The administration of 0.5 gm. thyroiodine per day for 3 days resulted in a fall to the normal level, after which the calcium again rose. Thyroiodine, however, had no effect on the serum calcium of normal animals. In another dog the thyroid and three parathyroids were removed. At first the serum calcium rose and then from the 25th day after the operation it fell to 25 per cent. below the normal value and persisted at this level. This decrease is ascribed to a hypoparathyroidism consequent upon the removal of three parathyroid glands, the remaining gland being unable on its own to maintain a high calcium level. Parhon and Derevici (1926) and Parhon, Derevici and Derevici (1930) extirpated the thyroid in dogs and found that there is a tendency for the serum calcium to decrease. The average value for twenty normal animals was 11.5, after thyroidectomy (six animals) the serum calcium decreased to 10.7 mg. per 100 c.c. Administration of thyroid substance had no effect. As the result of further experiments they came to the conclusion that the thyroid to a certain extent antagonises the development of hypocalcaemia after parathyroidectomy. Maxim and Vasiliu (1928) obtained large increases in serum calcium several days after thyroidectomy in dogs. In one case the calcium rose from 13.3 to 41.0 mg. per 100 c.c. 6 days after the operation. Werner (1929 *b*) found an increase of 19 per cent. in the serum calcium of thyroidectomised sheep. In one animal, in which the thyroid had been removed incompletely 2 years before, the calcium level was 10 mg. per 100 c.c. Eight months after extirpation of the fragment the calcium rose to 15.3 mg. Frei and Emmerson (1930) quote the results of Grüter who obtained a slight increase, about 9 per cent., in two thyroidectomised goats 51 days after the operation. Perelman (1925) found that extirpation of the thyroid gland in dogs and cats had no effect on the serum calcium. The same result is reported by Botchkareff and Danilova (1929) who removed the thyroids and internal parathyroids in sheep. Cheymol and Quinquaud (1932 *b*) removed the thyroid and internal parathyroids in dogs without any effect on serum calcium. Removal of the two remaining parathyroids led to a marked fall in calcium. Removal of the thyroid, the internal parathyroids and one external

parathyroid did not result in a fall in calcium. But subsequent extirpation of the remaining parathyroid, "*globuleuse et turgescente*," brought about a rapid and marked decrease.

Rabinowitch (1924) reported the effect on serum calcium of unilateral and bilateral lobectomy and subtotal thyroidectomy in twenty-six patients suffering from disease of the thyroid. No parathyroid tissue was found in the portion of the gland removed. In most cases a decrease in serum calcium was observed. This was explained as being due to trauma which the parathyroid glands suffer from manipulation during the operation or to temporary interference with the blood supply to the parathyroids. McCullagh (1928) reported a slight fall (0.3-1 mg.) in fifty of seventy-two cases of hypo- and hyperparathyroidism in which thyroid lobectomy or thyroidectomy was performed. He also ascribed this to trauma and disturbance in the blood supply in the parathyroid regions.

It is difficult to draw any definite conclusion from this mass of contradictory evidence. The majority of observers report a slight decrease or no effect after thyroidectomy. The results of Leites and of Grüter, who report increases after thyroidectomy, are based on only two animals in each case. The results of Maxim and Vasiliu and of Werner require confirmation.

It thus seems very likely that neither hyper- nor hypothyroidism, nor administration of thyroid or thyroxine, nor thyroidectomy influences to any significant extent the calcium level of the serum. The main action of the thyroid gland would appear to be concerned solely with the mobilisation of calcium from the bone depots, and its elimination through the excretory channels.

II. THE GONADS.

The more prolonged epiphysial growth of the bones after castration shown experimentally in many animals and evident in eunuchoids and castrated humans, suggested that the sex glands control the growth of the skeleton and indicated that they may play some part in calcium metabolism. Clinical observations indicate that in osteomalacia, especially during pregnancy, there occurs a marked softening of the bones, and the cure of osteomalacia by removal of the ovaries has frequently been reported. The early literature on the influence of the gonads on the phosphorus and calcium content of the body and skeleton and on phosphorus and calcium metabolism is reviewed by Korenchevsky (1923). In the light of recent work on the relationship between the gonads and the pituitary it is probable that the effects of castration on the skeleton is an indirect effect of the hypertrophy of the anterior lobe of the pituitary which occurs as a result of castration.

(1) THE EFFECT OF GONADECTOMY ON SERUM CALCIUM.

Reports in the literature in regard to the effect of gonadectomy on the calcium content of the serum are contradictory, especially, as might be expected, when the experimental animals used were rabbits. Leicher (1922) found in a woman a decreased serum calcium 2 months after ovariectomy. Blanchetière (1925) found a

large increase in four out of six cases of ovariectomy in women. A fall in serum calcium after removal of the ovaries in women was observed by Dalsace and Guillaumin (1925) and by Davanzo (1929), while Heyn and Haase (1925) reported a slight increase 2-8 months after ovariectomy. An increase in serum calcium after castration or ovariectomy in rabbits was observed by Leites (1924) and by Itoh (1930). Itoh found that after feeding dried ox testis to castrated male rabbits the serum calcium tended to rise. Injections of corpus luteum extracts had no effect on ovariectomised females. Werner (1929 *a*) reported large increases as the result of castration in guinea-pigs, sheep, and rabbits, and a slight decrease in dogs. Perelman (1925) found that gonadectomy in dogs and cats had no effect on serum calcium. Castration of parathyroidectomised animals, however, prevented the symptoms of tetany. Trifon (1929) obtained a slight increase in serum calcium, and Blinoff (1930) reported variable effects in guinea-pigs after castration. Suzuki (1931) found that ovariectomy in rabbits caused a fall at the end of the second week after the operation, after which there was a slight rise. Transplantation of ovary immediately after ovariectomy led, however, to a considerable decrease. Mirvish and Bosman (1927 *a*) obtained wide fluctuations, and Hogben and Charles (1932) could determine no significant changes in serum calcium after ovariectomy in rabbits. Frei and Emmerson (1930) reported that gonadectomy in cattle caused a tendency to decreased serum calcium values in males but increased values in females. Cheymol and Quinquaud (1932 *a*) state that extirpation of testes or ovaries is without effect on the serum calcium of dogs. Shapiro and Zwarenstein (1933) removed the ovaries or testes in *Xenopus laevis* (the South African clawed toad). Ovariectomy caused a persistent fall (17-24 per cent.) in the calcium content of the serum in females as soon as 2 months after the operation. In males there was no effect after 3½ months' castration but a fall (16 per cent.) after 6 months' castration. Females in captivity showed a progressive retrogression or involution of the ovaries and this was accompanied by a gradual decrease in serum calcium (Zwarenstein and Shapiro, 1933). Other evidence (see Section III) suggested that the ovary controls the level of calcium in the serum through some other endocrine organ, *e.g.* the parathyroids or adrenals.

(2) INJECTION OF GONADIAL EXTRACTS.

Mirvish and Bosman (1927 *a*) prepared alcoholic extracts of cows' ovaries which produced a fall in blood calcium on injection into rabbits. The calcium depressing principle was present in liquor folliculi, residual ovary, corpus luteum and placenta. The effect was the same in males, and in normal, pregnant or ovariectomised females. In women similar results were obtained; a fall occurred 12-24 hours after injection but the dose required was very much larger (Mirvish and Bosman, 1927 *b*). They inclined to the view that the oestrus-producing hormone was not the agent responsible but a different hormone present in the extract, which acts by inhibiting the parathyroids. Testicular extracts prepared in the same way also caused a fall in serum calcium of rabbits (Mirvish and Bosman, 1929 *b*). Reiss and Marx (1928) injected commercial preparations of ovaries into four rabbits and obtained a decrease

in serum calcium. Frei and Emmerson (1930) showed that there was a slight fall in serum calcium on injection of a commercial oestrin preparation (progynon) into cows. Dixon (1933) found that injection of extracts of residual ovary or corpus luteum into dogs produced no appreciable change in the serum calcium. Pregnancy and pseudo-pregnancy produced no demonstrable change in serum calcium 8 days after copulation in rabbits when the corpus luteum was assumed to have reached a reasonable degree of activity. Injection of crystalline trihydroxyoestrin sufficient to produce uterine distension and vaginal cornification in ovariectomised parathyroidectomised rats did not produce any marked difference in serum calcium from controls. Calcium determinations in rats at oestrus and dioestrus showed no appreciable difference. It thus seems that oestrin and corpus luteum hormone cause no significant changes in serum calcium in rabbits, dogs and rats. The earlier work of Luckhardt and Goldberg (1923) suggested that the hormone concerned in the production of oestrus lowers serum calcium. Parathyroidectomised dogs were treated with sufficient calcium to prevent tetany. During oestrus the animals exhibited a recurrence of all the symptoms of tetany. Frei and Emmerson (1930) found in cows that the calcium content of serum is somewhat higher in oestrus than in interoestrus. Maturation of the follicle and oestrus is accompanied by increased serum calcium. Immediately after ovulation there is a sharp fall. In the corpus luteum phase a slight and gradual rise takes place.

Zwarenstein and Shapiro (1934) found that extracts of ovarian tissue minus corpus luteum (Parke, Davis and Co.) significantly raise the lowered level of the serum calcium in ovariectomised toads and also raise the level of serum calcium in normal toads above the normal level.

(3) SERUM CALCIUM IN MENSTRUATION AND PREGNANCY.

Consistent variations in serum calcium have not been found at menstruation. Sharlit, Corscaden and Lyle (1927) and Matters (1929) reported a premenstrual rise in serum calcium and a menstrual fall in the calcium level. Boynton and Greisheimer (1931) found that there is a tendency for the serum calcium to be highest in the premenstrual period and lowest in the menstrual period. Their results suggest further that women show a cyclic variation in blood calcium which is absent in men. Rittmann (1924) estimated the serum calcium of seventy-seven women during menstruation. In 16.9 per cent. there was no change, in 40.2 per cent. an increase, and in 42.9 per cent. a decrease. Only a third showed increases or decreases exceeding 0.5 mg. per 100 c.c. Heyn and Haase (1925) and Malmud (1924) found irregular changes during menstruation. Watchorn (1926), Bock (1928), Close and Osman (1928), Allen and Goldthorpe (1929) and Spiegler (1931) observed a steady level during menstruation. Okey, Stewart and Greenwood (1930) observed a tendency to low values a few days before menstruation and higher values from 8th–15th day of the menstrual cycle. Kylin (1924) found an increased serum calcium during menstruation and high calcium values in cases of amenorrhoea. Boynton and Greisheimer (1932) examined ten cases of dysmenorrhoea. The mean range of serum calcium, while lower than that found in a group of women with

normal menstrual periods, was not significantly lower. The lowest mean serum calcium occurred in the rest period of the menstrual cycle.

There is some evidence which suggests that a decrease in serum calcium occurs at the menopause (Malmud and Mazzocco, 1923; Heyn and Haase, 1925). Blanchetière (1925), however, found an increase in two out of four cases of women at the menopause.

It has been rather generally observed that the calcium content of serum diminishes in the later months of pregnancy (Widdows, 1923; Bogert and Plass, 1923; Bokelmann and Bock, 1928; Bauer, Albright and Aub, 1929; Hellmuth and Timpe, 1930; Adler, 1931; Spiegler, 1931; Merritt and Bauer, 1931; Watchorn and McCance, 1932). The mechanism underlying this change is obscure. It was generally assumed that it was due to the increased demand for calcium by the foetus. Schönig (1928) and Adler (1931) show that this is improbable on theoretical grounds. Schönig draws attention to the fact that in spite of large losses of calcium during lactation a drop in serum calcium does not occur. Sjollem (1928) estimated that a cow which delivers 10 litres of milk per day excretes five times as much calcium as is contained in the total amount of blood in the animal. One litre of milk contains 12 to 13 times as much calcium as 1 litre of blood. Bauer, Albright and Aub (1929) investigated in a case of pregnancy whether the relatively large amount of calcium ordinarily excreted on a very low calcium intake could be used during pregnancy to meet the foetal demands for calcium. The subject excreted almost the same amount of calcium (on the low calcium diet) whether she was supplying a small amount of calcium to the foetus in the 5th month, a large amount in the 8th month or none at all as in the 2nd month after delivery. The calcium excretion was the same as if she had been non-pregnant. Thus calcium excreted on a low calcium diet is not available for the foetus. Adler (1931) suggested that the hypertrophy of the anterior lobe of the pituitary which is associated with pregnancy influences the thyroid and parathyroids so that calcium is retained in the tissues. Rodecurt, Koenig and Regensburger (1928) presented evidence which does not support this hypothesis. They found that the calcium content of the fluid in cantharides blisters, which they assumed to have the same composition as tissue fluid, is decreased during pregnancy. Although Adler's hypothesis in its original form cannot be accepted, it seems likely that an explanation of the change in the serum calcium level during pregnancy will be found on the lines suggested by him. McIsaac (1928) investigated calcium metabolism in the pregnant rabbit. Little or no difference in serum calcium was found between the early pregnant and non-pregnant animal, 7-10 days before parturition there was a fall and 1 day before a further and sudden fall to a minimum occurred. He suggests that pituitary activity at the close of pregnancy may account for these changes.

In addition to a decrease in total serum calcium a marked change in the normal distribution of the various forms of calcium also occurs. Bokelmann and Bock (1928), using the compensation dialysis method, found that the absolute values for dialysable calcium remained about the same throughout the greater part of pregnancy but a rise occurred in the last month. Hellmuth and Timpe (1930) observed

no alteration in the dialysable calcium during pregnancy. Cantarow, Montgomery and Bolton (1930) determined the calcium content of blood serum and of cerebro-spinal fluid. They assumed that the cerebro-spinal fluid calcium content is an accurate physiological representation of the diffusible calcium of the serum. During the course of pregnancy the diffusible calcium increased slightly and the non-diffusible calcium decreased strikingly. The ratio of the diffusible to non-diffusible calcium increased steadily, reaching a maximum in the first stage of labour. These findings were confirmed by Watchorn and McCance (1932) who obtained increased values for ultrafiltrable calcium during pregnancy. These changes are independent of the reduction in serum protein. They point out that the increase in the ultrafiltrable fraction of calcium may be of considerable biological significance because everything that passes through the placenta must presumably be in the ultrafiltrable form. The question in regard to the significance that can be attached to measurements of cerebro-spinal fluid calcium as an accurate representation of diffusible calcium cannot be gone into here. An excellent account will be found in Cantarow's book (1933). Searle and Michaels (1933) determined the calcium content of cerebro-spinal fluid serum and an ultrafiltrate of blood serum in 80 normal subjects. A close agreement was found between cerebro-spinal fluid and filtrate calcium and they conclude that over the normal range of serum calcium values the spinal fluid calcium is a close measure of the diffusible calcium of the blood.

(4) SERUM CALCIUM IN RELATION TO THE OVARIAN CYCLE IN BIRDS AND AMPHIBIA.

Riddle and Reinhart (1926) found that the serum calcium of female pigeons rose during the pre-ovulation phase to a value double that of the resting period. This high level was maintained during the greater part of the ovulation period and was succeeded by a post-ovulation fall to the normal resting level. These changes occupied in all about 10 days of the reproductive cycle. The active secretion of the egg shell begins only about 15 hours after the egg leaves the ovary, but the beginning of the increase in calcium dates approximately 108 hours before ovulation or a total of 123 hours before the egg shell begins to form. The high serum calcium is thus in no wise correlated with the large calcium need for shell formation and they inclined to the view that the very high calcium values accompanying each ovulation period is an expression of increased parathyroid activity. The rise and fall in calcium level in the fowl was found by Macowan (1932) to be associated with distinct histological changes in the parathyroids. Sun and Macowan (1930) showed that the serum calcium of White Leghorns rises considerably when egg laying commences, maintains a high average level during the laying period, to fall again when moulting commences. Buckner, Martin and Hull (1930) attempted to correlate the serum calcium of laying White Leghorns with the successive stages in the deposition of the albumin and shell. Determinations were made on nine sexually mature hens. For two which were inactive the figures were 17.8 and 19.1 mg. per 100 c.c. For a third which was moulting the serum calcium was 11.3 mg. In three birds a shell-less egg was present in the isthmus. These gave

values of 23.2, 12.9 and 24.5 mg. For two with partly-formed shells the values were 23.6 and 17.6, and for one with a fully-formed shell 17.6 mg. Charles (1931 *b*) found that the mean value for six Indian Game females before laying was 10.44 mg., and for six immature White Leghorn females 10.28 mg., the maximum deviations being 9.3 and 12.2 mg. For six Leghorns, taken from a flock which was laying, the figures varied between 10.8 and 18.3. Charles and Hogben (1933) recorded the determinations made upon female White Leghorns of the same flock kept under the same conditions before and after the onset of laying. The calcium content of the serum of sexually immature White Leghorn pullets was found to be 11.4 mg. per 100 c.c., and that of laying hens during the interval between oviposition and the next ovulation was 17 mg. When an egg was present in the oviduct the serum calcium varied between 10.5 and 28.5 mg. It is difficult to compare the results of Hogben and Charles with those of Riddle and Reinhart because, although Hogben and Charles give figures for mature active hens with no egg in the oviduct, they give only one figure (18.96 mg.) for mature but inactive birds. Two figures given by Buckner, Martin and Hull (1930), 17.8 and 19.1 for two inactive sexually mature hens suggest that there is no difference between the calcium levels of active or inactive sexually mature hens. Hogben and Charles draw attention to the fact that active elimination of calcium by the secretory activity of the shell gland may be associated with a blood calcium level which is much higher than that which is found when shell secretion is not in progress. They suggest that the presence of the egg in the oviduct results in the mobilisation of calcium in the tissues and that the distension of the oviduct reflexly stimulates the parathyroids. This suggestion is supported by results obtained when the oviduct was artificially distended with paraffin. They record, too, that in one of two birds which was not laying a calcium value of 26.76 mg. was observed, and in this bird the oviduct was found to be distended with fluid. The low values obtained are not necessarily due to the rapid elimination of calcium during the secretion of the shell. It was pointed out above that in spite of large losses of calcium during lactation in mammals, a drop in serum calcium does not occur. As a matter of fact, high values in the bird are characteristic of the period during which the shell is actually being formed, and low values were found to be more typical of the stage immediately preceding oviposition when the shell is complete, that is at the end of ovulation. An alternative hypothesis to explain the results on birds and largely supported by results in Amphibia to be described below, can be put forward. The difference between sexually immature hens with immature ovaries and a low calcium level and sexually mature birds (active or inactive) with mature ovaries and a higher calcium level may be an expression of pituitary activity. It seems likely that the effect of the pituitary on the ovaries and on the calcium content of the blood are concomitant but independent activities, the latter being probably due to indirect stimulation of the parathyroids. Both ovulation and the very high calcium values accompanying each ovulation may be due to increased activity of the pituitary at this time. Possibly the distension of the oviduct reflexly stimulates the parathyroids through the intermediation of the pituitary giving rise to the exceptionally high calcium values. Zwarenstein

and Shapiro (1933), working on *Xenopus laevis*, have shown that the serum calcium of female toads is lowest in autumn and early winter and highest in late winter and early spring, *i.e.* during the rainy and breeding seasons. The serum calcium remains at a high level throughout the spring and summer months. Up to the beginning of the breeding season there is a striking correlation between the state of the ovaries and the serum calcium. There is a gradual increase in both ovary weight and serum calcium, the highest level for both being reached in July, the beginning of the breeding season. After this the ovaries decline and lose weight as the ova are extruded in large numbers during the breeding season, and this loss of weight continues during the summer months. The serum calcium, on the other hand, after a slight fall, is maintained at a high level during this time. The changes in the serum calcium level of *Xenopus* are, except for the time relations, generally similar to the changes which occur in pigeons at the time of egg laying (Riddle and Reinhart, 1926). In spite of the fact that the eggs of *Xenopus* are not enclosed in a lime shell, the calcium content of the serum reaches its highest level during the breeding season, a fact which agrees with the absence of correlation between blood calcium and shell formation in birds. The relationship between the pituitary on the one hand and ovarian activity and serum calcium on the other will be discussed below.

(5) SEX DIFFERENCES IN THE CALCIUM CONTENT OF THE SERUM.

No significant sex differences in calcium content have been found in man by most observers. Boynton and Greisheimer (1931), however, determined the serum calcium of seventeen women and eleven men and found that the serum calcium level for women was lower (0.30 ± 0.0282 mg.) than for men. There was also a greater daily variation and a greater absolute range in women than in men. In other animals Mirvish and Bosman (1929 *a*) and Leites (1924) found no difference in rabbits; McIsaac (1928) found a larger serum calcium in male than in female rabbits, and Riddle and Reinhart (1926) found the reverse to be the case in pigeons. The latter observers point out that the issue is complicated by the very great rise in blood calcium of female pigeons during ovulation. A true sex difference can, therefore, only be looked for in immature birds. They give some figures for young birds showing a higher calcium level in males. The animals were killed at different seasons: the males at a period of high calcium level, and the females at a period of low calcium level. The authors do not regard their results as conclusive. Frei and Emmerson (1930) found that male calves have a lower serum calcium than females, while in adults the reverse is the case. Charles (1931 *b*) reported data with reference to sex differences in serum calcium and magnesium in *Lepus*, *Gallus*, *Acanthias* and *Yasus*. In rabbits the serum calcium was higher in males than in females, but the difference was not definitely significant. In fowls, the results uncomplicated by seasonal or reproductive variations, showed that in Indian Game the serum calcium was 12 per cent. lower in males than in females, the two series of calcium determinations being discontinuous. In White Leghorns the calcium content of the serum was 9 per cent. lower in males than in females, the difference being

definitely significant. In the Cape dogfish (*Acanthias*) the serum calcium was 3 per cent. lower in females than in males, but the difference was not significant. In the Cape crawfish (*Jasus lalandii*) no significant difference was obtained between males and females. In an earlier paper Charles (1931 *a*) reported that male *Xenopus* have a serum calcium 23 per cent. lower than females. This striking difference was confirmed by Zwarenstein and Shapiro (1933 *a*). The highest figure which Charles gives for females, the mean of nine determinations made in the hot season (February in South Africa), is 9.82 mg. calcium per 100 c.c. Zwarenstein and Shapiro, however, found that this figure represents actually the lower limit of the range of variation exhibited by female toads under natural conditions. The greatest difference in serum calcium between male and female toads occurred in August soon after the beginning of the breeding season with a serum calcium for females 46 per cent. higher than that of males. Charles (1931 *b*) points out that the two species of land vertebrates which show the most marked differences in serum calcium and magnesium possess well-defined secondary sexual characteristics; in the fowl, plumage, comb, spurs, etc., and in the toad, difference in size and the presence of anal labia in the female. In the two marine species studied the magnesium content of the serum appeared to be of greater significance than calcium particularly in connection with the reproductive cycle.

To recapitulate, an endocrine relationship between the gonads and calcium metabolism is by no means well established in mammals. There is some evidence that a decrease in total serum calcium and an increase in dialysable calcium occur during the later months of pregnancy, but this is probably due to an indirect effect of pituitary activity at this time. In Amphibia the results suggest that the ovary, directly or indirectly, controls the calcium content of the serum. Other evidence indicates that a relationship between the reproductive cycle and calcium metabolism exists in birds and Amphibia.

III. THE PITUITARY.

A relationship between the pituitary gland and calcium metabolism is indicated by the well-established effects of hypophysectomy and administration of anterior lobe substance on the growth and ossification of the bones in animals and by the striking skeletal changes in acromegaly and pituitary gigantism in man. Medi-greceanu and Kristeller (1911) found that calcium is retained in acromegalics. Injection of saline suspensions of anterior lobe into acromegalics led to a loss of calcium immediately following treatment, the positive calcium balance being converted into a negative one. Nakazawa (1928), however, reported that subcutaneous injections of commercial extracts of anterior and posterior lobes have no effect on the calcium and phosphorus excretion in urine and faeces. Waldorp (1926) studied four cases of acromegaly with increased basal metabolism. In three cases the serum calcium was decreased. In one case of acromegaly studied by Mirvish and Shapiro (1933) the serum calcium was high, 12.3 mg. per 100 c.c. An increased calcium content of serum was found by Leicher (1922) in two cases of dystrophia-adiposo-

genitalis. Houssay and Mazzocco (1922) observed a drop of 11 per cent. in the serum calcium of hypophysectomised dogs. Mazzocco (1927) and Gerschman (1931) found that hypophysectomy in dogs had no effect on the calcium content of plasma. Koster and Geesink (1929) removed the pituitary in young and adult dogs. The growth of the teeth was inhibited and the epiphyses remained open much longer in the operated than in the control animals. The serum calcium after total hypophysectomy was lower than in the controls. A definite fall, however, was obtained in only one of the three operated animals.

Leicher (1922) obtained a fall in serum calcium after administration of commercial posterior pituitary preparations in man. Davies, Dickens and Dodds (1926) found that injections of pituitrin led to a rise in the serum calcium of rabbits. Teel and Watkins (1929) observed a slight fall after injection of an alkaline extract of anterior lobe in dogs. This result, however, could not always be repeated.

Putnam, Benedict and Teel (1929) produced experimental acromegaly in dogs by the daily administration of aqueous extracts of ox anterior lobe over a period of 14 months. In addition to the generalised splanchnomegaly a skeletal overgrowth with hyperostosis resulted. There was an increase of about 11 per cent. in the serum calcium of the experimental animal. Frei and Emmerson (1930) have shown that there is a slight rise in serum calcium 8 hours after injection of a commercial anterior pituitary extract. They cite some experiments of Gortner who obtained an increase of 22 per cent. after injection of anterior lobe extract into two goats. Hogben and Charles (1932), in a series of carefully controlled experiments on female rabbits, showed that injection of fresh saline suspensions of ox pituitary produced a prolonged fall in serum calcium of normal and ovariectomised animals. After sexual excitement there was a fall of serum calcium in the female rabbit beginning within 4 hours after coitus, whether ovulation occurred or not. These results suggest that the pituitary produces a fall in serum calcium independently of its effect on the ovaries.

Cannavo (1932) failed to establish any significant changes in serum calcium after subcutaneous injections of prolactin into men, dogs and rabbits. On the other hand, a marked increase in blood magnesium occurred. The latter finding is of interest in the light of some results of Hogben and co-workers on the effect of hypophysectomy on the magnesium content of serum in *Xenopus laevis*. Charles (1931 a) obtained a drop of 27 per cent. after total hypophysectomy and a drop of 29 per cent. after removal of the anterior lobe alone. Subsequent work (Hogben, Charles and Slome, 1931) showed that the decrease after hypophysectomy occurred only during the hot season (February in South Africa). In July and September the serum magnesium of operated animals was higher than that of controls, so that the magnesium content of serum of hypophysectomised toads may be higher or lower according to the season of the year. It is thus necessary to speak for the present with some caution regarding the effects of pituitary removal on the magnesium content of serum. Dixon (1933) found that injections of anterior pituitary extracts into rats, sufficient to cause luteinisation of ovaries, caused no change in serum calcium.

The contradictory nature of the evidence reviewed above in regard to the effect of hypophysectomy or administration of pituitary extracts on the serum calcium in mammals precludes the possibility of arriving at any definite conclusion.

The work of Hogben and Slome (1931) and of Charles (1931 *a*) opened up a new field for an investigation of the problem. Removal of the pituitary gland in mammals is relatively an operation of extreme difficulty. On the other hand, removal of either the anterior lobe alone or both lobes in *Xenopus laevis* (Hogben and Slome, 1931) can be carried out in about half a minute with a negligible mortality and a high degree of accuracy, and the animals survive the operation for 2 years and longer. The characteristic colour response of the animal serves as an infallible indication as to the completeness of removal of either one or both lobes of the pituitary (Hogben and Slome, 1931). Conclusive evidence that the pituitary has a functional relation to calcium metabolism is lacking unless the effects of operative removal can be brought into harmonious relationship with the effects of injection and implantation. In *Xenopus* a complete investigation of this kind is possible in a striking manner. The pars tuberalis in *Xenopus* consists of a single triangular-shaped median lip attached to the superior margin of the pars anterior and inserted by a tapering process into the cleft extremity of the tuber cinereum (Hogben and Slome, 1931). It is ordinarily removed more or less completely when the anterior lobe is excised. As its tip adheres to the tuber cinereum the whole of the pars tuberalis does not always come away with the pars anterior and the remaining fragment may regenerate. To avoid confusion it must be emphasised that in the following discussion the term anterior lobe is used to include both the pars anterior and the pars tuberalis.

Charles (1931 *a*) showed that removal of either the anterior lobe alone or of both lobes in *Xenopus* caused a persistent fall in serum calcium, 37 and 26 per cent. respectively. The number of samples was not sufficient to permit of the conclusion that the difference between total removal and anterior lobe removal was significant. It was concluded that the anterior lobe is the main if not the only part of the pituitary which determines changes in serum calcium. In a later communication Hogben, Charles and Slome (1931) found that the fall in serum calcium after hypophysectomy is detectable throughout the year and is thus independent of seasonal variations. They concluded that "the relationship of the pituitary to calcium metabolism is complex and that both lobes exercise some influence, direct or indirect, upon calcium metabolism, since the fall in calcium content following on removal of the anterior lobe alone is greater than the fall consequent upon removal of both lobes." This was subsequently confirmed by Shapiro and Zwarenstein (1933), who suggested that the difference can be explained on the assumption that the anterior lobe normally maintains a high calcium level and the posterior lobe normally depresses serum calcium. It was found that injection of antuitrin (Parke, Davis and Co.) into completely hypophysectomised animals caused a rise and injection of pituitrin (Parke, Davis and Co.) a fall in serum calcium. When the pars tuberalis regenerates, as occurred in a number of animals in which the anterior lobe had been removed incompletely, leaving behind a small portion of the pars

tuberalis, the serum calcium returned to the normal level concomitantly with the reappearance of the white background response. This suggested that it is the regenerated pars tuberalis which caused the rise, and that it is only when this part of the pituitary is removed with the pars anterior in anterior lobe extirpation that the animal becomes permanently and maximally dark and a persistent fall in calcium occurs.

Gonadectomy caused a persistent fall in the calcium content of the serum (Shapiro and Zwarenstein, 1933). Since hypophysectomy leads to a striking retrogression of the ovaries (Hogben, 1930; Hogben, Charles and Slome, 1931; Zwarenstein and Shapiro, 1933 *a*; Shapiro and Shapiro, 1933) the effects of pituitary removal on serum calcium might have been due to a secondary effect through the ovaries. In the regenerated animals, however, the ovaries remained completely involuted but the serum calcium rose to normal (Shapiro and Zwarenstein, 1933). Anterior lobe removal alone also led to complete involution and yet to a low calcium level. Thus the same extreme degree of ovarian retrogression was associated with both a high and a low serum calcium. In the case of total removal of the pituitary or of castration the presence of a severely involuted ovary on the one hand and the total absence of ovarian tissue on the other were both associated with the same calcium level. These facts show that the pituitary does not control the serum calcium level through the ovaries and indicates that both the pituitary and the gonads indirectly influence calcium metabolism through some other endocrine gland, *e.g.* the parathyroids or adrenals. The results of Shapiro and Zwarenstein (1933) suggest that the influence of the pituitary on ovarian activity and its effect on serum calcium are concomitant but independent activities, and that the pars tuberalis and the posterior lobe exert antagonistic effects on both colour change and serum calcium. For final evidence on this point the effect of injections of extracts of pars tuberalis is necessary. The result obtained with injection of antuitrin, which in normal animals caused maximal contraction of melanophores on a black background and led to an increase in the low serum calcium level of hypophysectomised toads, is suggestive.

IV. THE ADRENALS.

Numerous investigators have sought to establish a relationship between the adrenal glands and calcium metabolism. The results of injections of adrenaline on the calcium content of blood have provided a mass of contradictory evidence from which no definite conclusion can be drawn. On the other hand, the results of extirpation experiments indicate that the cortex may play a part in the regulation of the calcium level in the blood, and now that an active principle of the cortex has been isolated, more definite evidence on this point will become available.

(i) EFFECT OF ADRENALINE ON SERUM CALCIUM.

Quest (1909) in dogs, Schiff and Peiper (1920) in children, and Elfer and Kappel (1920) in a patient suffering from osteomalacia, have reported that injections of adrenaline led to an increased excretion of calcium and a negative calcium balance.

Pulay and Richter (1926) found the total blood calcium and the ultrafiltrable calcium to be increased as the result of injections of adrenaline into rabbits. This was accompanied by a more or less severe acidosis. Jacobsohn and Rothschild (1927) established a slight increase in serum calcium in man, and Horsters (1930) obtained the same result in rabbits. A slight drop in serum calcium after injection of adrenaline has been reported by Leicher (1922), Billinghamer (1922), Hetenyi and van Gaal (1931) in man, Lamelas (1930) in cats and Woringer (1924) in dogs. Lamelas ascribes his results not to the action of adrenaline but rather to the large amount of fluid which was introduced with the adrenaline and retained. No definite effect was found by Mayer (1922) and Lawaczek (1928) in man. Lawaczek confirmed the finding of Pulay and Richter in regard to the increase in ultrafiltrable calcium. Raiha (1932) estimated in rabbits the calcium content of aqueous humour which he regards as an ultrafiltrate of blood. Adrenaline caused a fall in the dialysable fraction a few minutes after injection and a rise in the organically combined calcium fraction of blood serum. Variable effects on serum calcium in man were reported by Brems (1927 *a*), Castex and Scheingart (1928) and Hermann (1932). Bomskov (1932) assumed that the evidence in regard to the calcium depressing effect of adrenaline is well established. He suggested that the primary fall in serum calcium after injection of parathormone is due to stimulation of the adrenals and secretion of adrenaline. Both the assumption and the hypothesis, however, rest on insecure experimental evidence. It is doubtful whether adrenaline has a direct effect on serum calcium in view of the fact that it lowers inorganic phosphate and tends to produce acidosis. Blum, Delaville and van Caulaert (1924) found that in every case of acidosis, however produced, ultrafiltrable calcium is relatively increased. Earlier work by Vollmer (1923 *a, b*) suggests an explanation of the contradictory results reported. Vollmer claimed that adrenaline causes a rapid diphasic variation in various blood constituents. In man a definite decrease in inorganic phosphate and an increase in serum calcium occurs 5–15 min. after injection of adrenaline. This rapid phase is followed by a slow phase in which the inorganic phosphate rises and the calcium falls. The changes in calcium follow the changes in phosphate. Vollmer (1923 *c*) also found that about 15 min. after injection of adrenaline there is an increased excretion of acid in the urine followed after 30 min. by a decreased excretion. The second phase is maintained for some time. Dresel and Katz (1922) reported that injection of adrenaline caused a fall in serum potassium half an hour after the injection. During the next half hour the potassium increased until it exceeded the normal value and then gradually returned to normal. Allan, Dickson and Markowitz (1924) found that the administration of adrenaline to fasting dogs caused changes in the excretion of inorganic phosphate in the urine similar to those caused by administration of insulin or by injection of sugar, namely, an initial decrease followed by a larger increase. According to Vollmer, these various effects of adrenaline occur in a definite sequence. In the first rapid phase the inorganic phosphate falls; this is followed by a rise in calcium and a drop in potassium. As a result oxidative processes are inhibited and this leads to an acidosis. In the second phase all these effects are reversed and finally lead to an

alkalosis. The change in phosphate and hydrogen-ion concentration (Gottschalk and Pohle, 1922) is regarded as being of basic importance in determining all the other actions of adrenaline. Although Vollmer's hypothesis affords a ready explanation of the various effects of adrenaline reported it is by no means well established, and doubt has been cast on some of his results (Dresel and Wollheim, 1924).

According to Zondek (1923) the autonomic nervous system exercises an influence on calcium metabolism. Wollheim (1924) reported that stimulation of the splanchnic nerves after cutting the vagus in dogs decreases the calcium content of blood and increases that of the tissues. Adrenaline (Dresel and Wollheim, 1924) has the same action. Stimulation of the vagus after section of the splanchnic nerves has the opposite effect. Berg, Hess and Sherman (1928) obtained a definite decrease in the calcium level of the blood after cutting the right or left splanchnic nerves or after removal of the coeliac and superior mesenteric ganglia in dogs. Section of the vagi led to a rise in serum calcium. Lamelas (1930) removed the sympathetic system in cats from the stellate to the pelvic ganglia on both sides, but this had no effect on the calcium content of the serum. Section of the right or left splanchnic nerves was likewise without effect. Observations on the prolonged injection of fluid containing adrenaline suggested the possibility that the phenomena observed by Berg and co-workers may have been due to dilution resulting from a fall in blood pressure and the passage of fluid from the tissue spaces into the blood.

(2) ADRENALECTOMY.

Kisch (1924) reported that the serum calcium of two adrenalectomised rabbits first decreased and then rose above normal. Rohdenburg and Krehbiel (1925) in rats and Keitel (1926) in dogs found increases in serum calcium after extirpation of the adrenals. Lucas (1926) and Viale and Bruno (1927) found no noteworthy change in dogs. Baumann and Kurland (1927) pointed out that most investigators had been studying the effects of trauma and shock rather than those of adrenalectomy. This is evident from the fact that even when the adrenals were removed in two stages their animals usually died in less than 48 hours after removal of the second gland. Dogs and cats usually survive a clean operation for several days and rabbits several weeks. Accessory adrenals occur more frequently in rabbits than in cats and dogs. Baumann and Kurland found little change in the plasma calcium or phosphate of most of their adrenalectomised cats. In three animals, however, unusually high calcium values were found, 13.3, 14.5 and 16.2 mg. per 100 c.c. They suggested that the adrenals normally exert an inhibitory influence on the rate at which many organs function. Removal augments the rate. The parathyroids are also stimulated as a result of adrenalectomy and this causes the very high calcium values found. Taylor and Caven (1927) reported that removal of both adrenals produced a definite rise in the serum calcium of cats within 3-5 hours after the operation. Of thirty-six operated animals thirty-two showed a rise. Four adrenalectomised dogs showed a pronounced rise from 12 to 15.6 mg. per 100 c.c. in one case, and from 11 to 14.8 mg. in another. Removal of the alimentary canal, kidneys or spleen had no effect on serum calcium. Tying the

adrenal veins had the same effect on serum calcium as adrenalectomy. Removal of one gland and freezing the other but leaving the blood supply intact was without effect. In most cases there was a rise in blood concentration accompanying the hypercalcaemia, but this was insufficient in degree to account for the increase in serum calcium. Removal of one adrenal and the medulla of the other was without effect. The results of combined parathyroidectomy and adrenalectomy suggest that removal of the adrenal affects the calcium level only when there is some parathyroid tissue present. Rogoff and Stewart (1928) removed the adrenals in dogs. This resulted in most cases in either a definite increase in serum calcium or a tendency to rise. In two or three animals there was no change.

It thus seems definitely established that removal of the adrenals leads to an increase in serum calcium. This effect is probably due to removal of the cortex. One should therefore expect a fall in serum calcium on injection of cortical extracts into normal animals.

(3) INJECTION OF CORTICAL EXTRACTS.

Taylor and Caven (1927) found that extracts prepared from the cortex of ox adrenals depressed the serum calcium in rabbits from 15 to 30 per cent. Mirvish and Bosman (1929 *a*) prepared an alcoholic extract of the cortex which lowered the blood calcium in rabbits about 30 per cent. The maximum effect occurred 24 hours after injection and the normal level was regained after 48 hours. Extracts of muscle, pancreas, spleen, kidney and brain had no significant effect. These results cannot be finally accepted until the effect of cortin has been investigated on other animals.

V. THE PANCREAS.

A relationship between the pancreas and calcium metabolism was suggested by the observations of von Moraczewski (1897), Van Noorden (1907) and Kahn and Kahn (1916), who found that diabetes is associated with a negative calcium balance.

(1) SERUM CALCIUM IN DIABETES AND AFTER INJECTION OF INSULIN.

A number of workers have reported low serum calcium values in diabetes accompanied by acidosis: Kahn and Kahn (1916), Loeper and Bechamp (1910) and Percival and Stewart (1926). Kylin (1926 *a*, 1926 *b*, 1927) investigated the blood calcium level in a large number of diabetic patients. As a rule high calcium values were found, but most of his figures do not show any striking or significant deviation from the normal. Normal values in diabetes were observed by Rothschild and Jacobsohn (1926) and by Brems (1927 *b*).

The effect of insulin on the calcium content of serum has been extensively investigated. Briggs, Koechig, Doisy and Weber (1924) found that the serum calcium remained essentially unchanged after administration of insulin to dogs. Similar results were obtained by Farquharson and Tibbetts (1931) in man. Davies, Dickens and Dodds (1926) and Schmidt and Ssaatschian (1927) found increases up to 30 per cent. in the serum calcium of rabbits in hypoglycaemic convulsions,

a result which Culhane (1927) failed to confirm. Brougher (1927) has reported large increases in the serum calcium of normal and parathyroidectomised dogs following the administration of insulin. Reed (1929) found that insulin administered to normal and to parathyroidectomised dogs not in tetany will increase serum calcium about 10 per cent. When in tetany insulin will relieve the symptoms usually completely. Brougher criticises the results of Briggs and co-workers because of the delay in time in making the estimations. Harrop and Benedict (1924) demonstrated that the administration of glucose or glucose and insulin to normal individuals brought about a fall in serum inorganic phosphate accompanied by a decreased excretion of phosphorus in the urine which is followed by an increased compensatory elimination, but the serum calcium did not change significantly. Blatherwick, Bell and Hill (1924) observed that insulin administered to normal individuals before glucose ingestion caused a marked decrease in the inorganic phosphorus of blood plasma and in the rate of excretion of phosphorus in urine. Glucose alone, however, gave variable results. Under certain conditions insulin may cause at first a slight increase in plasma and urinary phosphate followed by the customary drop. Ellsworth (1930) found that when blood phosphate is suddenly lowered by administration of glucose and insulin the serum calcium rises. This was not due to an anhydraemia and decreased blood volume such as has been described by Drabkin and Edwards (1924) and Drabkin and Shilkret (1927) after injection of insulin into animals. The anhydraemia observed by them occurred only in association with hypoglycaemia, whereas the increased calcium values obtained by Ellsworth occurred during periods when the blood sugar was also increasing. Blood chloride estimations also showed that there was not sufficient change in blood volume to account for the rise in blood calcium. Farquharson and Tibbetts (1931) also found that administration of glucose and insulin was constantly followed by a fall in serum inorganic phosphate. Sometimes, but not always, increases in serum calcium, similar to those found by Ellsworth, occurred.

Staub, Gunther and Frohlich (1923) also observed a decrease in the inorganic phosphorus of serum and urine in a normal dog and in a patient with diabetic coma after insulin injection. The serum calcium of the dog decreased slightly and that of the patient increased about 23 per cent. Kylin (1926 *a*) found a constant decrease in serum calcium after injections of large amounts of insulin into diabetic patients. Meyer-Bisch (1925) in dogs had found that insulin led to an increase in the calcium content of the lymph in the thoracic duct. Kylin therefore suggested that insulin causes the calcium to pass from blood to lymph, but he admits that there may be other explanations. Meyer-Bisch and Gunther (1924) studied the effect of oral administration of glucose and fructose on serum calcium in normal and diabetic individuals. The results were rather confusing. In eight patients, most of whom were under 50 years of age, slight increases in serum calcium were observed; in four further cases, three of whom were over 50 years of age, a decrease resulted; and in two cases there was no effect on administration of fructose. Glucose had no effect. No change in serum calcium was observed on the administration of glucose or fructose to normal subjects.

Raiha (1932) found that 10 min. after the injection of small doses of insulin into rabbits a fall in the calcium content of aqueous humour (which he regarded as a true blood dialysate) occurred. There was no consistent effect on serum calcium. The fall in the calcium content of aqueous humour was followed an hour later by a rise. With larger doses a rise occurred 10 min. after injection. Adrenaline caused a fall in aqueous humour calcium within a few minutes after injection. He suggests that the initial decrease after small doses of insulin was due to an increased secretion of adrenaline. This masks the true insulin effect. He comes to the conclusion that as a result of insulin injection the organically combined calcium fraction of blood serum decreases while the dialysable fraction is increased. Adrenaline has the opposite effect. The results are, however, vitiated to a large extent by the fact that rabbits were used as experimental animals, that the effect of diet and repeated bleedings were insufficiently controlled, and that the results are few and by no means definite.

The evidence on the whole suggests that the administration of insulin or of insulin and glucose causes in most cases a rise in serum calcium. It is highly probable that this is not a direct effect but that the primary action of insulin with or without glucose is upon the blood phosphate and that the rise in serum calcium when it occurs, is a secondary phenomenon. In diabetes there is a tendency to acidæmia and this is another factor which probably contributes towards an increase in serum calcium.

(2) PANCREATECTOMY.

A definite effect on calcium metabolism as a result of removing the pancreas has not been established. Falta and Whitney (1908) found that pancreatectomy was associated with an immediate and rapid elimination of calcium. Meyer-Bisch and Bock (1927) observed in most cases a decrease in serum calcium after extirpation of the pancreas in dogs, a result which was not in agreement with the earlier finding of Perelman (1925) in dogs and cats and which was not confirmed by Cahane (1931) in dogs.

VI. THE LYMPHOCYTOGENIC ORGANS.

(1) THE THYMUS.

(a) *Thymectomy.*

Although there is little reliable evidence that the thymus has any true endocrine function, the data in the literature suggest that the organ may be of significance in calcium metabolism. Embryologically, a functional relationship between the thymus and parathyroids seems likely, and it is significant that most of the effects of thymectomy that have been reported are related to calcium metabolism and impairment of ossification.

As early as 1858 Friedleben claimed to have obtained abnormal development and softening of the bones in young animals after thymectomy. Numerous investigators subsequently reported that removal of the thymus caused a condition closely related to, if not actually identical with, the rickets of human beings. In 1919 Park

and McClure published a critical review analysing in detail the experiments of previous workers. The claims to have established a definite relationship between thymectomy and the abnormal development of bones were shown to be unfounded. Park and McClure stress the difficulty of complete removal of the thymus in animals on account of the occurrence of detached lobes about the thyroid. Their own carefully controlled experiments showed that thymectomy in young dogs is not followed by detectable symptoms. The thymus is not essential to life and is not necessary for the normal process of ossification. A number of previous workers experimenting on frogs, guinea-pigs, rats and rabbits (see Park and McClure, 1919) had arrived at the same conclusion. Recent work, Van Allen (1926) on rabbits and Andersen (1932) on rats, has served to confirm these results.

(b) *Injection of extracts.*

An impetus to renewed investigation into the relationship between the thymus and calcium metabolism was given by the publication in 1929 of Nitschke's paper on the effects of an extract of thymus on the calcium and inorganic phosphate content of the blood. In an introduction the literature on the effect of thymectomy on calcium metabolism is reviewed. He points out that the contradictory results reported may have been due to injury of the thyroid and parathyroids during the operation. Macciotta (1925) reported that after thymectomy in rabbits, besides the increased calcium excretion in the faeces and urine a considerable increase in serum calcium occurred—values up to 80 mg. per 100 c.c. Crema (1925) injected large doses of thymus extract intravenously into rabbits and dogs. He observed a marked diminution of serum calcium in three rabbits and in one of two dogs. These experiments were, however, insufficiently controlled and very incompletely described. Leites (1924) reported that thymectomy in young dogs led to changes quite similar to but less profound than those which follow parathyroidectomy. Growth was inhibited and on the 3rd day after the operation a decrease in serum calcium set in until on the 17th day it was 30 per cent. below normal. Administration of a commercial extract of thymus caused an increase to normal. From the 54th day after extirpation the serum calcium gradually increased until 10 days later it reached a value 10 per cent. below normal, at which level it remained. Feeding thymus to normal animals had no effect. Nishimura (1928) found that extirpation of the thymus or thymus and thyroid caused a considerable decrease in serum calcium. Nitschke (1929 a) obtained an active substance from calf's thymus by acetic acid-alcohol extraction. Subcutaneous injection into adult rabbits led to a 50 per cent. decrease in serum calcium. This was accompanied by generalised tetanic spasms followed in most cases by the death of the animal. No food was given on the day before the collection of the first sample of blood and during the experiment which lasted in most cases for 2 days. The effect of repeated bleedings was controlled. Injections of extracts of muscle, liver or kidney had no effect. He also prepared an active extract from thymus which caused a drop (27–63 per cent.) in the inorganic phosphate content of the serum. The calcium depressing substance is soluble in water, while the substance which affects the inorganic phosphate is insoluble in

water but soluble in ether or acetone. Nitschke suggests that the thymus acts as an antagonist to the parathyroids and draws attention to the close resemblance between the effects of injection of thymus extracts and the symptoms and changes in blood calcium and inorganic phosphate in infantile tetany. Spleen extracts and extracts of lymph glands gave the same result as thymus extracts (Nitschke, 1929 *b*). In lymph glands the calcium depressing substance was present in smaller quantities than in thymus or spleen. On the basis of these results Nitschke suggested that the failure to demonstrate any effects after extirpation of the thymus is explicable on the assumption that its function may be immediately compensated for by other lymphoid tissues. Riddle and Krizenecky (1931), in an excellent discussion of thymectomy and thymus function, came to a similar conclusion. They write: "Approached by several methods of investigation the thymus has revealed some good evidence of endocrine and other functions but it is a most singular fact that the existence of no such function has been definitely found nor even clearly confirmed by the removal of the organ." They suggest the existence of other tissues capable of performing one or other thymic function. It thus seems that, apart from the difficulty of complete removal of all thymic tissue, attempts to investigate thymus function by removal of that organ is doomed to failure on account of the existence of vicariously functioning tissue. Evidence for an endocrine function of the thymus can only, therefore, be obtained from experiments on the effects of administration or implantation supplemented by clinical data and other indirect evidence. By these methods suggestive indications of thymic function can be obtained, but, since a complete demonstration of the endocrine function of a gland must include evidence showing that any effects of removal is compensated for by administration of extracts of that gland, it follows that such evidence is unobtainable in the case of the thymus.

Nitschke (1929 *c*) drew attention to the resemblance between the effects of injections of thymus extracts and the syndrome of spasmophilia in children. Urine of spasmophilic and of normal children (aged 10 months to 2½ years) was extracted by the same method as was used for glands. A marked fall was obtained on injecting spasmophilic urine extract into rabbits. Only a slight fall occurred with extracts from normal urine. The urine extract from a case of latent spasmophilia depressed the serum calcium level from 13.0 to 8.0 mg. per 100 c.c. Nitschke suggests that spasmophilia may be due to hyperactivity of lymphoid tissue (thymus, spleen and lymph glands) and that this system is an antagonist of the parathyroids.

Reiss, Winter and Halpern (1929) obtained a decrease of 14–35 per cent. in the serum calcium of rabbits within 3–24 hours after injection of thymus or spleen extracts. The total and bone calcium was estimated in mice after injection of thymus or spleen extracts daily for 1–2 weeks. The total calcium increased 17 per cent. and bone calcium 30 per cent. as compared with controls. Muscle and liver extracts gave negative results. Wu (1930) found that implantation of rabbit thymus or spleen into rabbits caused a greater depression of the serum calcium level than implantation of liver or kidney. The inorganic phosphate content of the blood was unaffected. Asher (1930) prepared a thymus extract which stimulates the growth

of young rats. The growth-promoting principle (thymocrescin) promotes calcium retention. Zenklusen (1932) is doubtful whether the results obtained by Nitschke can really be ascribed to hormonal action. He points out that the injection caused severe symptoms ending in several cases with the death of the animal. On the other hand, injection of thymocrescin has no injurious effects. He investigated the effect of thymocrescin on serum calcium in rabbits (two animals only) but failed to establish any effect. Nitzescu and Benetato (1932) repeated and confirmed Nitschke's results on rabbits. They obtained a definite fall in serum calcium and a large decrease in inorganic phosphate. In the dog Nitschke's extract caused a hypocalcaemia which was especially evident after previous extirpation of three parathyroids. The increased serum calcium after parathormone injection into dogs did not occur on injection of thymus extract and parathormone at the same time. Miwa (1932) reported that after thymectomy in rabbits the serum calcium showed a tendency to drop but regained the normal level 8 days after the operation. Injection of thymus or of spleen extracts (Nitschke's method) caused a fall in serum calcium. Scholtz (1932) found that thymocrescin caused a definite drop in the serum calcium of rats. The hypercalcaemia induced by injection of parathormone can be reduced by injection of thymus extract. The normal calcium level in rats is 11.0 mg. per 100 c.c. Injection of 15 units of parathormone daily for 8 days caused a rise to 23.2 mg. On the other hand, parathormone injection plus 1.5 c.c. thymocrescin for 8 days caused an increase to only 14.2 mg. Spleen extracts had no results. After parathormone administration there was a slight decrease in the calcium content of bone. With parathormone plus thymocrescin there was a tendency to an increased calcium content of bone. Thymocrescin alone has no effect on bone calcium. Histological examination showed that injection of parathormone caused lacunar resorption of bone similar to that which occurs in osteitis fibrosa. Parathormone plus thymocrescin gave a histological picture which indicated increased bone formation. Scholtz (1933) obtained a decrease of only 1.0 mg. per 100 c.c. in the serum calcium of dogs after injecting thymocrescin. Scholtz concludes that all thymus preparations influence serum calcium and that his own data and that of previous investigators suggest a relationship between the thymus and calcium metabolism which is fairly well established. The evidence as a whole suggests that the thymus acts as an antagonist to the parathyroids. According to Nitschke hyperfunction of the thymus plays an important part in the pathogenesis of tetany. Asher's thymocrescin leads to calcium retention, while the work of Aub and his collaborators has shown that parathormone results in a negative calcium balance. The thymus contains a growth-stimulating principle, while Robinson and Thompson's (1932) results indicate that the parathyroids contain an anti-growth factor. Thymus injections decrease while parathormone increases serum calcium. In regard to the effect on serum calcium, dogs are more resistant than rats to injections of thymus extracts, and rats are more resistant than dogs to injections of the parathyroid hormone. These reciprocal relations and the increased susceptibility of dogs to thymus injection after removal of three parathyroids is evidence in favour of the existence of a thymus-parathyroid antagonism.

Harris (1930) summarises several facts which indicate that the thymus is involved in calcium and phosphorus metabolism. Although a number of points which he adduces in favour of such a conception has been questioned or has not been confirmed, the new evidence presented above supports his suggestion that the thymus is concerned in promoting calcification.

(c) *Relation of the thymus to the secretion of egg envelopes in birds.*

Several investigators have suggested that the thymus may be connected with calcium metabolism in birds. In 1911 Soli, in a rather inaccessible paper (his results are described by Riddle and Krizenecky (1931) and by Greenwood and Blyth (1931)), reported that 15–20 days following thymectomy in mature fowls the calcium metabolism becomes deranged as indicated by the production of eggs in which the shell was soft or absent; after a further period of about 40 days there was a return to normal calcium metabolism and the production of eggs with normal shells. Soli's results have been widely quoted, but recent data have failed to confirm them. In December, 1929, Ackert and Morris and Morgan and Grierson simultaneously published abstracts of results that agreed in the main points regarding thymectomy in chicks and young fowls. The former observers could detect no constant differences between thymectomised and control birds in weight of eggs, strength of egg shells, calcium carbonate in egg shells, appearance and thickness of egg shells, fertility and hatchability of eggs. In a later publication Morgan and Grierson (1930) described their results in more detail. They removed the thymus in young fowls and later repeated the operations in order to search for and remove any remaining or regenerated thymus. In this way four complete thymectomies were performed. Regeneration occurred in the other cases. No noticeable effect upon egg production was observed. There was no sign that calcium metabolism was in any way affected by thymectomy. Greenwood and Blyth (1931) reported the results of thymus extirpation in mature fowls, and their work is thus more strictly comparable with that of Soli. In two of the operated birds a small fragment of thymus was subsequently found and in only one was the operation completely successful. Their data indicate that removal of the thymus in the hen led neither to lowering of the calcium content of the blood nor to the production of eggs deficient in shell or egg membranes. Nine immature fowls were thymectomised, but the eggs subsequently produced were normal. As mentioned above, Riddle and Krizenecky (1931) suggested that failure to establish the existence of an endocrine or other function of the thymus by thymectomy was due to the presence and hyperplasia of supplementary or vicariously functioning tissue. In birds this extra-thymic tissue is represented by the bursa fabricii, the two post-branchial bodies, the lymph glands and other lymphoid organs and areas adjacent to the thymus. The thymus and bursa were completely extirpated in seventeen pigeons. No influence on body growth was noted and females produced normal eggs. They point out that the early removal of the thymus and bursa may have permitted hyperplasia in the remaining scattered tissue with thymic function before the beginning of egg production. Thymectomy in birds as in mammals thus leads to no detectable effect.

One experiment on the effects of thymus administration to birds suggests a functional relationship between that gland and the secretion of shell and other egg envelopes. Among a large number of doves and pigeons Riddle (1924) noted certain females that showed various reproductive disorders such as asymmetric and small eggs, small yolks, deficient egg envelopes and infertility. Among such birds were some whose eggs had yolks of normal size but were deficient in albumin and shell. Birds showing these abnormalities were subsequently found to have extremely small thymus glands. Doses of desiccated ox thymus were daily fed to these birds and the amount of albumin and shell substance was greatly increased during and following administration. Dosage with other desiccated tissues had no effect. Riddle concluded that the thymus produced a substance having a highly specific action on the oviduct of birds and probably of all those vertebrates which secrete egg envelopes.

(d) *The thymus and the calcareous bodies of Schwammerdam.*

Schnitzer (1930) reported the effect of thymectomy on the calcareous bodies which are found on either side of the vertebral column in close association with the spinal ganglia in frogs. The morphology of these bodies was first described by Lenhossek in 1886. Vincent-Loison (1926) described the contents which consist of a milky fluid which contains fine crystals of triple phosphate and calcium carbonate. The physiology of these structures is obscure. After several months' captivity and starvation the crystals disappear almost completely. They seem to bear no relation to seasonal changes, the reproductive cycle, or diet. Schnitzer took X-ray photographs of these calcium deposits in *Rana fusca* before and after thymectomy and in a series of non-operated controls. The animals survived the operation for 9 weeks. Fourteen days after the operation the calcareous bodies of thymectomised frogs decreased in size, and after 8 weeks they had completely disappeared. Control animals showed no change even after 10 weeks.

The evidence, as a whole, suggests that a relationship between the thymus and calcium metabolism exists, but most of the work has been done on rabbits, and the results, except perhaps for the carefully controlled experiments of Nitschke and the few results on dogs and rats, must be viewed with caution. More experiments on animals other than rabbits, which are notoriously unsuitable for investigations on calcium metabolism, unless the conditions are rigidly controlled, are necessary. Definite proof that the thymus is an endocrine organ is still lacking.

(2) THE SPLEEN.

In the light of Nitschke's results with lymphocytogenic organs the relationship between the spleen and calcium metabolism is of interest. Hall and Ablahadian (1925) found that intravenous injection of a splenic extract into rabbits caused a considerable rise in blood calcium. After removal of the spleen in two rabbits the calcium dropped almost 50 per cent. but returned to normal after administration of the splenic extract. Krumbhaar (1926) states that "unfinished work of our own tends to confirm them to a certain extent," and they suggest a possible relationship

between the spleen and the parathyroids. Underhill and Gross (1929) failed to establish any notable change in the serum calcium level in rabbits after splenectomy or after intravenous injections of spleen nucleoprotein. Nishimura (1928) claims that splenectomy in animals increases serum calcium. Nitschke (1929 *b*), Reiss, Winter and Halpern (1929), Wu (1930) and Miwa (1932), as already noted above, reported that the spleen contains a substance which has a depressant action on blood calcium in rabbits. Scholtz (1932) found no change after injection of splenic extract into rats. Brougher (1930), on the basis of the results of Hall and Ablahadian and of Krumbhaar, investigated the effect of splenectomy on thyroparathyroid-ectomised dogs. Removal of the spleen in normal dogs caused a fall in serum calcium; laparotomy or other operations produced no change. Administration of desiccated spleen raised the serum calcium of splenectomised dogs but was ineffective in parathyroidectomised animals. These results, it is concluded, point to a specific action of the spleen on calcium metabolism. A number of parathyroid-ectomised dogs which had recovered from tetany were splenectomised. A further decrease in the calcium level resulted and two animals developed tetany. Recovery in parathyroidectomised dogs was thought to indicate that the spleen had to some extent taken over the functions of the parathyroid. Miwa (1932) published the results of an extensive investigation on the relationship between the spleen and calcium metabolism in male rabbits. His results, although suggestive, are by no means definite or clear-cut, and his conclusions are, on the whole, based on insufficient, and in some cases, contradictory evidence.

VII. THE PINEAL.

Only one reference to the relationship between the pineal and calcium metabolism was encountered in the literature. De Candia (1931) investigated the effect of intravenous injection of pineal extract in dogs and men, but consistent results were not obtained. In five dogs there was an increase in serum calcium to four times the initial value, but in two animals a fall occurred. In eight men, there was a decrease in three, a rise in four and no effect in one.

VIII. SUMMARY.

1. Neither hyper- nor hypothyroidism, nor administration of thyroid or thyroxine, nor thyroidectomy influences to any significant extent the calcium level of the serum. The main action of the thyroid gland would appear to be concerned solely with the mobilisation of calcium from the bone depots and its elimination through the excretory channels.

2. An endocrine relationship between the gonads and calcium metabolism is by no means well established in mammals. There is some evidence that a decrease in total serum calcium and an increase in dialysable calcium occurs during the later months of pregnancy, but this may be due to an indirect effect of pituitary activity at this time. In Amphibia (*Xenopus laevis*) the results suggest that the ovary, directly or indirectly, controls the calcium content of the serum. Other evidence indicates that a relationship between the reproductive cycle and calcium metabolism exists in birds and Amphibia.

3. The contradictory nature of the evidence in regard to the effect of hypophysectomy or of administration of pituitary extracts on serum calcium in mammals precludes the possibility of arriving at any definite conclusion. The results in Amphibia (*Xenopus laevis*) indicate that there is an antagonistic relationship between the two lobes of the pituitary in regard to the regulation of the serum calcium level and afford evidence in favour of the existence of an endocrine relationship, direct or indirect, between the pituitary and calcium metabolism.

4. The results of injections of adrenaline on the calcium content of the serum have provided a mass of contradictory evidence from which no definite conclusion can be drawn. On the other hand, the results of extirpation experiments and of injections of cortical extracts indicate that the cortex probably plays a part in the regulation of the serum calcium level.

5. The administration of insulin or of insulin and glucose produces in most cases a rise in serum calcium. It is highly probable that this is not a direct effect, but that the primary action of insulin with or without glucose is upon blood phosphate and that the rise in serum calcium occurs as a secondary phenomenon. A definite effect on calcium metabolism as a result of pancreatectomy has not been established.

6. The evidence suggests that a relationship between the thymus and calcium metabolism exists, but most of the work has been done on rabbits and the results, except perhaps for the carefully controlled experiments of Nitschke and a few results on dogs and rats must be viewed with caution. Definite proof that the thymus is an endocrine organ is still lacking.

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THE BACTERIOPHAGES

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CONTENTS.

	PAGE
I. Introduction	332
II. The characteristics of the bacteriophage particles	333
III. The mechanism of lysis	336
IV. The specific relationships between phages and bacteria	339
V. The multiplicity of phages	342
VI. Some ecological considerations	345
VII. The alleged production of bacteriophage from normal bacteria	348
VIII. Conclusion	349
References	349

I. INTRODUCTION.

THE simplest way to demonstrate the activity of a bacteriophage is to take a small fragment of pig or horse faeces, emulsify it in broth and from the suspension prepare a bacteria-free filtrate by filtration first through paper and then through any standard bacteriological filter. In nine cases out of ten this filtrate will contain bacteriophage particles whose activity can be demonstrated on a culture of dysentery bacilli, particularly of the Shiga type. A very young broth culture of dysentery bacilli showing a just detectable turbidity is inoculated with a few drops of the filtrate and incubated along with a control culture without the added filtrate. At first the two cultures show the usual steady increase in turbidity, but in a few hours the inoculated tube begins to lag behind the control and then rapidly becomes limpid. All the bacteria have been dissolved and concurrently an agent has appeared in the fluid which is capable of producing the same lysis when added to a fresh dysentery culture. This agent is a bacteriophage (or shortly, phage) and in such a lysed culture it is present in high concentration. A hundred-millionth of a cubic centimetre will usually be sufficient to induce the characteristic changes in a further growing culture. At each passage the same enormous increase in the amount of phage takes place, and the process can be continued indefinitely.

(Phage activity can be demonstrated even more strikingly on a solid nutrient medium. Agar plates are uniformly spread with a few drops of a young broth culture of dysentery bacilli, and, when the surface has dried, successive 10-fold

dilutions of a lysed broth culture are spread over the surface, one dilution to each plate. The plates are again allowed to dry, incubated and examined the next day. On the first four plates there will probably be no growth whatever in the region spread with the bacteriophage dilutions, except for an occasional well-isolated "resistant" colony. The next two plates will show a characteristic "worm-eaten" appearance, and, as a rule, the sixth on which a few drops of a 1 : 1,000,000 dilution have been spread will show well-isolated bacteriophage "plaques." These take the form of circular clearings in the uniform layer of bacterial growth covering the agar surface. The central zone of the plaque is quite clear, but the peripheral region forms a more or less steeply shelving edge of partially lysed bacteria. By suitable means it can be shown that a large amount of phage is present in the region of each plaque. Under proper conditions the number of plaques obtained is at least approximately proportional to the amount of phage spread, and the commonest method of titrating phage activity is to count the plaques obtained from a known dilution of the preparation in question.)

Phenomena of this general type were first observed by Twort in 1915, rediscovered in more typical form by d'Herelle in 1917 and by him forcefully elucidated in a long series of papers and several books (1921, 1926, 1930). He strenuously supported the theory that they were due to the activities of an autonomous living micro-organism, a parasite of bacteria, and claimed that in many intestinal diseases the whole outcome of the disease depended on the interaction between the infecting bacillus and the corresponding bacteriophage. Perhaps because of the heterodox nature of these conceptions rather than from any fault in d'Herelle's logic, the majority of bacteriologists who then took up the problem followed Bordet and Ciuca (1920) in regarding the phenomena as of bacterial origin and not due to an extrinsic parasite. The controversy has since persisted with gradually diminishing intensity but without the appearance of any unanimity. With the steady accumulation of facts, however, the theories of intrinsic origin seem to have become progressively more difficult to maintain. One is certainly justified in stating that however agnostic they may be in regard to the nature of a phage, all workers manipulate and in practice think of it as an extrinsic virus-like agent. In the opinion of the present writer this pragmatic attitude is wholly justified by all the available data bearing on the more theoretical questions of the biological position of the phenomena.

It is the aim of this review to cover the more important contributions to the literature which have appeared since the publication of previous reviews of the subject by d'Herelle (1926), Bronfenbrenner (1928), Hadley (1928) and Burnet (1930), and to discuss particularly those aspects of bacteriophage phenomena which are of more general biological interest.

II. THE CHARACTERISTICS OF THE BACTERIOPHAGE PARTICLES.

There is little serious objection to the view that the active agent in lytic filtrates is particulate. The characteristic plaques which appear when sufficiently dilute phage is spread with a susceptible culture on agar, alone provide almost complete

proof of this. Working with broth cultures d'Herelle showed early that if equal amounts of phage diluted almost to the limit of its activity were distributed amongst say ten young broth cultures of the susceptible organism, only a proportion of the tubes would show lysis. These after incubation contained high-titre phage, those failing to lyse contained none whatever. This type of experiment has recently been elaborated by Feemster and Wells (1933), who, using statistical methods, find that the proportion of tubes showing lysis is precisely what would be expected if the active agent were particulate.

For the most part information as to the physico-chemical nature of the active particles has to be obtained in more or less indirect fashion, but recent work concerned particularly with the size of the particles has given some more definite results. Elford and Andrewes (1932) have applied the methods of filtration through graded collodion membranes, elaborated by Elford, to the problem using a comprehensive series of bacteriophages. They find that while the particles of any single pure phage appear to be of uniform size irrespective of the bacterial strain used in their production, there are great differences between different phages. Amongst the phages lysing dysentery bacilli there is one (S₁₃) whose particles are of the same size (8–12 $m\mu$) as those of the smallest known virus, that of foot and mouth disease. At the other end of the scale there is a very common group of phages whose particle size is estimated to lie between 50 and 75 $m\mu$ in diameter. Between these limits there are at least four other sizes of particle making an almost continuous series.

These results must be regarded as of the greatest theoretical importance. In the first place they indicate that bacteriophages cannot possibly be regarded as mere variants either of a single micro-organism or of some bacterial constituent but comprise a number of distinct types of agent. Secondly they indicate that for the larger particle types at least, the phage particles are far too large to be regarded as anything but organised bodies, and further they suggested the possibility that with these particular phages the particles could be demonstrated optically by the refined microphotographic methods now available. This has since been accomplished using Barnard's methods of photography by monochromatic ultraviolet light (Burnet, 1933 *b*). Photographs of the aggregates obtained when a phage of this type is treated with homologous antiphage serum show them to be composed of uniformly sized granules just at the limits of resolution for this method, *i.e.* around 50 $m\mu$ in diameter. Granules of the same size can also be photographed directly in concentrated preparations of the same phage without the use of antiphage serum.

Further confirmation of the relatively large particle size of these phages is provided by the work of Schlesinger (1932) in Bechhold's laboratory. Using a phage which is very closely similar to that used in Burnet's work he found that the particle size as deduced from the rate of sedimentation in a high-speed centrifuge was from 79 to 90 $m\mu$ in diameter. When the phage was grown at the expense of a culture of *B. coli* in a simple synthetic medium, high-titre filtrates showed an easily demonstrated Tyndall effect when exposed to a strong beam of light. The highest titre filtrates were visibly opalescent under ordinary illumination, and Schlesinger (1933) found that the intensity of the Tyndall effect was proportional to and could be used

to titrate the amount of phage present. These latter findings, which I can confirm, give another direct indication of the relatively large size of the particles concerned.

These more recent findings from the National Institute for Medical Research and from Bechhold's laboratory are in such general agreement that they must be regarded as supplanting the very discordant results of previous work on the particle size of the bacteriophages. There is nothing to be gained therefore in attempting to discuss the earlier results. It may be stressed, however, that Elford and Andrewes' results are completely incompatible with the interpretation placed on diffusion experiments by Hetler and Bronfenbrenner (1931). The latter authors consider that a phage preparation consists of particles of varying size on which is adsorbed an active principle of relatively low molecular weight corresponding to a particle diameter of not more than $0.4\text{ m}\mu$.

In regard to the physico-chemical constitution of the particles not much can be said. Several lines of evidence suggest, however, that in all probability bacteriophage particles are not very dissimilar in structure from bacteria and probably almost equally complex chemically. The particles under ordinary conditions migrate to the anode in an electric field (Todd, 1927; Burnet and McKie, 1930*a*; Natarajan and Hyde, 1930), over the whole range of pH within which they can remain active. Certain phages appear to be less definitely electronegative (Burnet and McKie, 1930*a*), and Kligler and Olitzki (1931) state that some phages when "purified" by adsorption and elution are amphoteric.

Specific antibodies against bacteriophages can be readily obtained by ordinary immunological methods, presumptive evidence of the presence of protein in their structure. The specific antigen can be shown to be at least largely on the surface of the particle, but it is also capable of existing in soluble form and can be detected in phage-free ultrafiltrates of lysed cultures (Burnet, 1933). There is slight evidence that the interaction between ultrafiltrate and the antiphage serum is due to a haptene (perhaps of carbohydrate nature) rather than to a complete antigen. Further indirect evidence of the presence of protein is provided by the fact that a well-marked antagonistic effect of Na⁺ and Ca⁺ ions on the viability of phages can be demonstrated (Burnet and McKie, 1930*a*). The analogous effects on many types of living cells are usually regarded as dependent on the protein present in the surface of the cell, although others regard the lipoids as being predominantly concerned. In any case the phenomenon seems to indicate the existence of a characteristically living surface. Some of the larger particle phages are rapidly inactivated and apparently disintegrated by strong urea solution (Burnet, 1933), a reagent whose chief effect is to denature proteins (Hopkins, 1930) and which acts similarly on certain bacteria and viruses. Another resemblance between phages and other living organisms is found in the inactivating effects of methylene blue and some other dyes in the presence of light (Perdrau and Todd, 1933). Finally it may be mentioned that a small proportion of bacteriophages are inactivated by trypsin (Schultz and Krueger, 1928) although most are highly resistant.

Now that the existence of relatively large particle phages has been established it should not be impracticable to obtain a sufficient bulk of washed phage particles to

conduct some direct microchemical investigations on their composition. There are fairly numerous accounts in the literature of "protein-free" phage preparations, but none of these has been of a sufficiently high phage titre to make the finding of any significance. Even a large-particle phage with the very high titre of 10^{10} particles per c.c. contains only about 2 mg. of phage substance per litre. Schlesinger (1933) has recently described a technique by which appreciable amounts of phage substance may be collected using a large-particle phage grown with *B. coli* in synthetic medium, and the results of microchemical studies on this material will be awaited with interest.

III. THE MECHANISM OF LYSIS.

The classical manifestation of bacteriophage activity, lysis of a growing broth culture, was interpreted by d'Herelle in simple terms on the basis of his parasitic theory of phage. (Each phage particle added to the culture invaded a growing bacterium, multiplied within it and eventually caused its more or less explosive disintegration, liberating at the same time a brood of 6-60 descendant phage particles which in their turn invaded fresh bacteria and continued the process until all the available bacteria had been lysed.) d'Herelle based this view on the fact that when very small amounts of phage were added to a growing sensitive culture the phage concentration at first increased in steps about 20-30 min. apart. After two or three such steps the increase followed a logarithmic course till near the time when complete visible lysis occurred.

This simple point of view has been severely criticised by many authors whose general objection is that there is no evidence that bacterial lysis and phage multiplication are so inevitably related as is assumed by d'Herelle's hypothesis. It is particularly stressed that at a period when the phage concentration is actively increasing there is no evidence of any change in the number of viable bacteria, lysis only occurring visibly when the phage concentration has reached its peak. This point is emphasised by Krueger and Northrop (1931), who are responsible for the most detailed quantitative study of the reaction that has been published.

They used an active staphylococcal phage and estimated the concentrations of phage and bacteria at various stages of the lytic process by accurate methods. Their results were reproducible and could be expressed in mathematical form, but it is not easy to interpret them in biological terms. Krueger and Northrop (1931) themselves have refrained from any attempt at such a biological interpretation, although they incline to the view that phage is a relatively simple unorganised agent.

Their chief findings may be summarily stated as follows. (Increase in bacteriophage concentration only occurs in the presence of growing bacteria, and the rate of increase is a function of the rate of bacterial growth. After a lag period of 15-30 min. the increase in phage concentration follows a logarithmic course until visible lysis of the bacteria appears, when there is a final fall. Most of the phage in this "logarithmic phase" is bound to the bacteria, a constant relationship between intracellular and extracellular phage being maintained. (Lysis occurs whenever the total concentration of phage reaches or exceeds a certain value per bacterium.) Fig. 1, reproduced from

their paper, allows the course of a typical experiment to be seen at a glance. Further experiments by Krueger (1931) on the primary union between phage and bacterium showed that both living and heat-killed bacteria took up the phage at initially equal rates. With living bacteria the union was reversible, and an equilibrium between "intracellular" and "extracellular" phage according to a simple partition relationship was established. With dead (heat-killed) bacteria the union was irreversible and resembled a typical absorption.

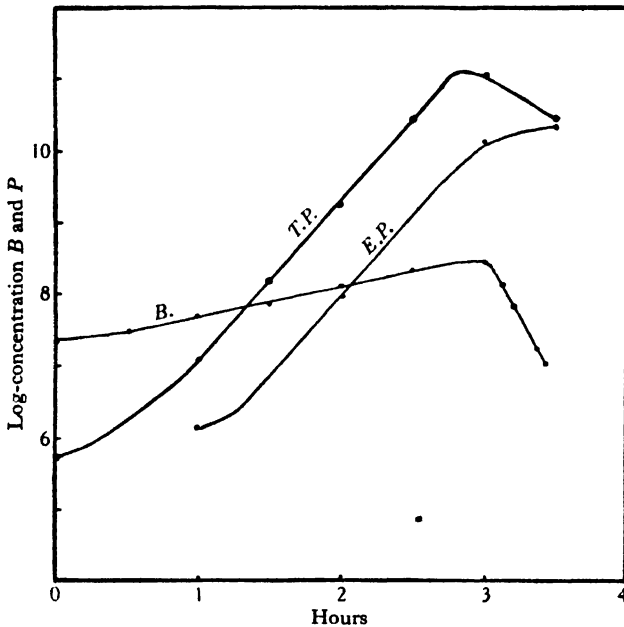


Fig. 1. Course of phage multiplication in relation to bacterial growth. Concentration of bacteria (*B.*) and total (*T.P.*) and extracellular phage (*E.P.*) shown logarithmically against time in hours. Redrawn from Krueger and Northrop (1931, Fig. 3, p. 225).

Krueger and Northrop (1931) confined their discussion of these results to a formulation of the kinetics of the reaction in mathematical terms. In any attempt to assess their more biological implications certain additional facts must be considered. In the first place Elford and Andrewes (1932) have shown that the phage used in these experiments has a particle size of 50–75 μ , *i.e.* it is far too large for the partition relationship found to have any simple physical meaning. All Krueger and Northrop's data are based on the interaction of large numbers of phage particles and bacteria, and their essentially statistical results do not give much information as to what takes place in the interaction between a single phage particle and a single bacterium. It is quite practicable, however, to obtain direct evidence as to the nature of this interaction using the method described by Burnet in 1929 *b*. The principle of the method is to distribute highly diluted phage in small volumes of growing sensitive culture so that each volume contains on the average one phage

particle. At frequent intervals the whole contents of a tube are spread on well-dried agar so that the number of free particles in the tube at the moment of spreading will be indicated by the number of plaques which develop on the agar. With this technique it is found that an active phage shows no increase in the number of plaques for a period of about 20 min. Then there is a sudden increase in numbers: a typical series of platings made at 1 min. intervals in a satisfactory experiment is 1, 0, 1, 2, 0, 1, 80, 0, 1, 120, 1, 230, 0, 100. There is a sharp discontinuity between the initial 0, 1 or 2 plaques and the subsequent large number in this case around 100 or 100×2 . The only interpretation which can be given to such an experiment is that favoured by d'Herelle. The phage particle has clearly for the first 20 min. been multiplying under some spatial constraint which is then suddenly released. When we combine this information with the fact that when a large excess of phage is added to multiplying bacteria in broth or on agar, visible lysis as judged microscopically or macroscopically occurs about 20 min. after the addition, one must admit that the liberation from spatial constraint can only be synonymous with the disruption of the invaded bacterium and the associated liberation of phage particles into the medium.

The quantitative results of Krueger and Northrop are wholly compatible with this point of view provided we make the not unreasonable postulate that (phage particles just liberated from a lysing bacterium are more active as judged by the kinetic method used for phage estimation in these experiments, than those which have been free for a longer period.) This assumption is required to account for the final apparent fall in phage titre.

(The morphological aspects of bacteriophage lysis have been fairly extensively studied both by ordinary bacteriological methods and by microcinematography.) The descriptions given by different authors vary considerably, but the discrepancies are probably to be accounted for mainly by differences in the type of bacteriophage studied. The commonest finding with bacteria of the coli-dysentery group is for the bacterium to swell slightly but otherwise appear essentially normal for 20–30 min. and then within a second to lose its outline and disappear. Bayne-Jones and Sandholzer (1933) have obtained cinematograph records of this disappearance and find that there is a sudden great increase in size, the bacillus assuming a globular form before it disappears. The final stage occupies less than a second and seems to represent a sudden general loss of interfacial tension at the bacterial surface. They find also that amongst the bacteria exposed to phage there is always a considerable proportion which cease to grow or slowly develop into bizarre forms without undergoing the typical sudden lysis. Similar happenings may be observed in lysing broth cultures. I have not infrequently found that a fairly sudden partial clearing occurs, but a mild turbidity persists for some hours. In some cases at least this turbidity can be removed by shaking the culture with chloroform: it seems probable that the final disintegration of the bacteria is determined by a number of factors of which intracellular multiplication of phage is only one.

(The formation of plaques on an agar-surface culture has not been studied in detailed quantitative fashion, but there is a general consensus of opinion that the process is essentially similar to that occurring in broth cultures.) A phage particle

comes into contact with a sensitive growing bacterium and initiates its lysis. With the disintegration of the organism the liberated phage particles diffuse in the surface film of fluid on the agar until they come into contact with further bacteria from which the process is continued. As the bacteria accumulate their rate of growth slackens and concomitantly the rate at which the phage can multiply. The size of the plaque produced therefore will be determined by (1) the diffusibility of the phage particles in the surface film, (2) the rate at which the lytic process takes place in each attacked bacterium, and (3) the period over which the bacteria continue to multiply and so remain susceptible to lysis. It will be evident from this that the size of plaque produced by a given phage will vary widely with the conditions, of which the most important are the concentration of agar in the medium, the temperature of incubation, the nature of the bacterial culture used and the thickness of the primary bacterial inoculum.

The number of plaques produced from a given inoculum also varies greatly according to the conditions present. The simplest statement of the position is that when all conditions are optimal each phage particle produces one plaque, any deviation from these conditions resulting in certain particles failing to initiate lysis. It must be confessed that this does not offer any explanation of the results obtained by Dreyer and Campbell-Renton (1933). They observed that the number of plaques produced is not directly proportional to the amount of phage used. If a standard volume of diluted phage gives 500 plaques, an equal volume of a 1 : 10 dilution gives approximately 80 plaques. This phenomenon has not yet been given any theoretical interpretation.

IV. THE SPECIFIC RELATIONSHIPS BETWEEN PHAGES AND BACTERIA.

It was recognised early that phages were more or less strictly limited in the range of bacteria which they are capable of lysing. A phagelysing dysentery bacilli is totally inactive against a *Staphylococcus* or a cholera vibrio and *vice versa*. On the other hand when a group of related bacteria such as the intestinal forms placed in the genus *Bacterium* by Topley and Wilson (1930), is studied with the aid of a series of appropriate bacteriophages very complex results are obtained. Certain phages are strictly limited in their activity to a single species or even to a few strains only of a species, while others can lyse practically every form included in the genus. All sorts of intermediate grades of activity also exist. A further apparent increase in complexity is introduced if one also includes the so-called "rough" (R) variants in the survey. These on the whole are susceptible to a wider range of phages than are the corresponding normal "smooth" (S) forms, but there are certain phages which act exclusively or much more actively on the S form. In such a group as the dysentery (Flexner) bacilli it is found that the S forms fall into five or six distinct serological groups, but that the R variants possess a new serological character which is common to them all. It is significant that a similar grouping is obtained by testing their susceptibility to various phages. Each of the S serological types has a distinctive behaviour toward phages, while the R variants are all identical in their reactions and

sensitive to a greater range of phages than any of the S strains (Burnet and McKie, 1930*b*).

There is another type of bacterial variant that is of great importance for bacteriophage theory. This is the "resistant" variant which appears following phage lysis of a sensitive culture. In many instances such a variant differs from the parent strain only by the fact that it is completely insusceptible to lysis by the phage in question. Serologically and in its behaviour toward other types of phage it is unaltered. In other cases more or less marked changes in other characteristics may accompany resistance, in particular a certain degree of roughness is common in the resistant derivatives of S strains. [For references see Hadley, 1928.]

Within the one group of bacteria, then, we find variations in susceptibility to phages which are related to (1) the nature of the species, (2) the smooth-rough alternation, and (3) the presence or absence of acquired resistance to certain phages. With a very occasional exception all these variations can be correlated with the adsorptive capacity of the bacteria for the phages in question. Any given strain adsorbs those phage particles which are capable of lysing it and fails to adsorb those which lack the capacity. In practice certain phages are much more readily adsorbed by sensitive bacteria than others, and there is a correspondingly greater ease in demonstrating the difference between susceptible and insusceptible strains. For at least the great majority of phages it appears certain that lysis is primarily determined by the ability of the phage to be adsorbed specifically to the surface of the bacterium.

The nature of the bacterial component responsible for this adsorption is not yet known with certainty, but the evidence indicates forcibly that it is closely related to and perhaps identical with the specific polysaccharide-containing antigens. The evidence for this is derived partly from the fact that in general bacteria possessing the same antigenic structure are susceptible to the same phages. There is, for instance, an extensive group of bacteria which possess in common an O (polysaccharide-containing) antigen but are otherwise largely dissimilar; it includes the typhoid bacillus *B. enteritidis* and the agents of fowl typhoid and bacillary white diarrhoea of chicks. All these are sensitive to one type of bacteriophage which is without action on any strains lacking this antigen, including the R variants of the sensitive S strains. The R variants of all the Salmonella group have a common antigen component and all show closely similar susceptibility to phages (Burnet, 1927). On the other hand, it is possible to obtain resistant variants which show no detectable antigenic difference from the sensitive parent strain, so that if phage reactions are primarily determined by the molecular configuration of the antigen they must be more susceptible to slight changes in this configuration than are the serological reactions (Burnet, 1929*a*).

Further evidence for the importance of the antigens of the bacterial surface is coming to light in recent work on the phage-inhibiting activity of filtered bacterial extracts (Levine and Frisch, 1933; Burnet, unpublished). It is possible to obtain extracts from autolysed bacteria which can inhibit the activity of those phages which are capable of lysing the strain in question. When a strain showing acquired resistance to a particular phage is used as the source of the extract, inhibition of this

phage does not occur though phages which are still active against the variant are normally inhibited. The soluble phage-inhibiting agent thus possesses the same specificity toward phages as is shown by the bacteria from which it is derived and it is reasonable to assume that it represents the actual surface component of the bacteria which determines the adsorption and subsequent lytic activity of the phage particle.

The soluble agents are completely inactivated by the homologous antibacterial serum and concurrently a typical precipitin reaction between serum and extract occurs. Cross reactions between different sera and extracts according to the ordinary methods of serological investigations indicate that the precipitation and the inactivation of the phage-inhibiting agent are two aspects of the same reaction, the specificity of which runs strictly parallel to the antigenic structure of the bacteria providing the extracts. In other words the precipitating antigen and the phage-inhibiting substance are identical and represent in a soluble form the somatic agglutinin and the substances responsible for binding phage to the intact bacterium. The specificity of this substance seems to be determined mainly or wholly by the specific polysaccharide haptene. It is noteworthy, however, that the purified carbohydrate as ordinarily prepared lacks the properties of the phage-binding substance and it remains to be determined whether the active agent represents a compound of polysaccharide with protein or is itself a relatively unstable polysaccharide from which the usually studied haptene has been produced by the chemical manipulations involved.

This question of the specific relationships between phages and bacteria has not been very extensively investigated but such evidence as is available is compatible with the above interpretations. The difference in susceptibility to phages of S and R variants is now generally recognised. The A type cholera phages of Asheshov *et al.* (1930) act only on typically S agglutinable vibrios while C type phages are more active against R strains. Denys (1932) has also noted that certain phages act only on S dysentery strains, others only on R, while others act on both forms. Hadley (1928) has discussed this question extensively but appears to lay undue stress on the occasional correlation of roughness with resistance. In a systematic study of the streptococci Lancefield (1933) found that susceptibility to a streptococcal bacteriophage was closely but not absolutely correlated with the possession of a particular carbohydrate antigen.

The specific relationship between bacteriophages and a particular antigenic component of the bacterial surface is of very considerable general interest. The relationship between the two is highly suggestive of the antigen-antibody relation and the fact that it is the same bacterial antigen which determines the specificity of the most important antibody and which offers the point of attack of the phage-particle can hardly be without significance. [The activity of a phage toward a given bacterium and the readiness with which it is adsorbed can often be greatly increased by appropriate passage, *i.e.* in all probability the phage particles become modified to "fit" the antigenic pattern of the bacteria more accurately.] The change may of course merely represent a selection of fortuitous variations, but it is at least thinkable that the actual process of passage has in some way impressed the new specificity upon the phage particle.

V. THE MULTIPLICITY OF PHAGES.

Supporters of both the opposing views as to the nature of bacteriophages have tended to assume that only one type of agent was involved. d'Herelle regarded phages as all belonging to one highly variable species *Protobios bacteriophagum* and all potentially interconvertible by appropriate passage. Bordet and Ciuca (1920) regarded lysis as a nutritive vitiation of bacterial metabolism and most other theorists have been content to discuss how one type of lytic agent might be derived from a normal bacterium.

Recent work, however, has been emphatic in demonstrating that a very wide range of agents must be included within the group of bacteriophages. Bail (1923) was the first to stress this point of view and his work on the differentiation of phages according to plaque size and cross-resistance tests remains the basis for any system of phage classification. Bail studied the phages acting on dysentery bacilli and his results have been confirmed and extended for this group by Morison (1932) and Burnet and McKie (1930*b*). Similar results have been obtained with cholera phages (Asheshov *et al.*, 1930) and with phages acting on certain *Salmonellas* and staphylococci (Burnet, 1930).

In discussing the question of phage diversity and systematic classification it will be convenient to concentrate almost exclusively on the series of dysentery-coli phages accumulated by Burnet, and used in his own studies with Miss McKie and also by Elford and Andrewes (1932) in their estimations of particle size. It is the only extensive series of phages described in the literature which has been systematically studied in regard to all the more important differentiating characteristics. It must be recognised, however, that the conclusions drawn are in no way exclusively based on this material. Bail's early studies were also concerned with an extensive material and with his collaborators he was able to show that the qualities of plaque size, resistance-provoking power and serological character provide the three primary criteria for any phage classification. Many other authors, *e.g.* Gratia and de Namur (1923), Bruynoghe (1924) and Bronfenbrenner and Korb (1925), have concerned themselves with the differences amongst bacteriophages lysing a single bacterial strain and assisted the gradual clarification of ideas.

(Amongst the phages capable of lysing a single strain, differences in the size of plaque produced may often be a sharply differentiating feature.) The plaques which appear when a crude phage-containing filtrate is tested on agar with a suitable bacterial culture may show a wide range of size. If fairly soft (1 per cent.) agar is used the range of diameters may range from a centimetre down to a barely visible pinpoint. (If material is taken from a well-isolated single plaque and after suitable dilution retested on a similar culture the plaques obtained will be found to be all of a similar size and appearance.) As in the similar technique of isolating a pure bacterial strain it is advisable to repeat the isolation from single plaques once or twice in order to be sure that the phage is "pure." (Each pure phage gives rise under standardised conditions to plaques of the same structure and of a definite range of diameters.) With some the size is very uniform, a size distribution

curve having a sharp almost central peak, others show a wider range the curve being flatter and usually asymmetrical with a more gradual slope on the "smaller" side.

When all the other conditions are uniform differences in the plaque size characteristic of two different pure phages must be related primarily to their diffusibility, i.e. the size of the phage particles. This inverse relationship between particle size and plaque diameter has been directly established by Elford and Andrewes (1932) who found that different types of phage showed particles ranging in size from 8-12 $m\mu$ for the large plaque phage S 13 to 50-75 $m\mu$ for a group of phages producing very small plaques. Schlesinger (1933) has recently studied some of the same phages by means of a centrifugation technique and has confirmed the existence of a wide range of particle sizes but gives absolute values which are considerably higher than those of Elford and Andrewes. These results at once dispose of the contention of d'Herelle that only one type of micro-organism is concerned in phage phenomena. It is impossible to conceive that an entity 10 $m\mu$ in diameter is merely a functional modification of another about 6 times this diameter.

The importance of particle size in the classification of phages is strengthened when it is considered in relation to the functional differences which they present. These concern, on the one hand, the range of activity of phages toward different bacterial species and more specifically toward different variants of an initially sensitive bacterial strain, and, on the other, their behaviour toward anti-bacteriophage sera.

When a sensitive culture is lysed by a pure phage, in most cases a secondary growth of a variant resistant to lysis by this phage appears. If this variant is now tested with a large series of pure phages some will be found still capable of lysing it, others fully active against the original strain completely fail to lyse the variant. According to Bail's method of classification all the phages which fail to lyse the resistant strain fall into the same resistance group as the phage which was used to provoke the appearance of the resistant variant. In practice this technique gives rise to many complexities, examples of which may be found in papers by Bail (1923) and Burnet and McKie (1933). The latter authors considered that a small number of "major resistance groups" could be delimited amongst the dysentery-coli phages, some of them comprising several minor resistance groups. This classification is to a large extent empirical and many difficulties had to be slurred over in order to include all the phages studied in these major groups, but such an arrangement seems to be the most useful until more knowledge is available in regard to the nature of induced bacterial resistance to phage.

The serological differentiation of phages gives more clear-cut results. When active bacteriophage filtrates are injected repeatedly into rabbits their sera develop the power to inactivate the corresponding phage. One may divide all the phages lysing dysentery-coli strains into a fairly small number (at least 11) of distinct serological groups. Such a group is defined as comprising all the phages which are significantly inactivated by an active antiphage serum produced by immunisation with any one of them. It was found that according to this criterion the serological

groups were sharply delimited. In no instance was any pure phage inactivated by antisera corresponding to phages of two or more serological groups. Within the group minor differences exist, an antiphage serum always inactivating the homologous phage more strongly than any other.

In these ways about fifty pure phages lysing dysentery and coli strains have been classified according to (1) plaque size and therefore particle size, (2) major resistance group, (3) serological type. From the results certain principles have emerged. The phages of a given serological group are always uniform in regard to plaque size and major resistance group, but phages of a given particle size may belong to any of the five major resistance groups while phages of the same major resistance group may include types of several different particle sizes. (If bacteriophages are to be divided into species the serological grouping seems to be the most satisfactory criterion.) The serological character of a phage is a definite intrinsic property of the active corpuscles. The resistance groups seem to depend on differences in the form which bacterial variation takes under the influence of phages and are only in rather indirect fashion indicative of differences in phage structure.

A further indication that the serological grouping divides phages into natural species has been obtained by the use of certain biochemical tests (Burnet, 1933). These are (1) the ability of phages to develop on media from which the Ca ion has been removed by the addition of citrate, (2) susceptibility to photodynamic inactivation by methylene blue and certain other dyes, and (3) susceptibility to inactivation by strong urea solutions. It has been well known that phages differ widely in their power to develop on citrate-containing media (Stassano and de Beaufort, 1925; Bordet, 1926; Asheshov, 1926), and an examination of the whole available series showed that three of the serological groups were completely inhibited on citrate media while two others were moderately inhibited. Schultz and Krueger (1928) first observed the inactivating effect of methylene blue on certain staphylococcal phages which Clifton (1931) later showed to be a photodynamic action. Different phages vary greatly in their susceptibility though all can be inactivated to some extent if the irradiation is intense enough. Comparative studies with the dysentery-coli phages available showed that for each serological group there was a characteristic degree of susceptibility. Similarly with the effect of strong urea solutions there was again a uniformity in the reactions of the phages in any one serological group with wide differences in susceptibility between different groups.

The conclusion is inevitable that as in all other assemblages of living organisms the process of evolution has tended to the emergence of distinct species of bacteriophage, at least as definite as bacterial species but like them labile in many of their properties and sometimes appearing to merge by way of rather rare intermediate types into other species. The reality of these species is strengthened by the fact that examples of a given serological type may be obtained in material from widely distinct geographical regions. All six of the types of dysentery phages isolated by Morison (1932) in India, G, H, J, K, L, M, for example, could be placed in their appropriate serological group by the use of antisera made against phages which had been isolated either in Australia or in England.

VI. SOME ECOLOGICAL CONSIDERATIONS.

If bacteriophages are independent micro-organisms parasitic on bacteria one would expect them to occur in nature particularly in material which regularly harbours a bacterial flora of relatively high concentration and approximately constant composition, and which can be infected by material from other similar sources. The situation in which these conditions are most completely fulfilled is the alimentary canal of gregarious vertebrates. The great majority of the bacteriophages described in the literature have been isolated from human or animal faeces, mixed city sewage and swine faeces are perhaps the most useful, and what little is known of bacteriophage ecology is limited to those phages which can multiply at the expense of the normal or pathogenic bacteria of the intestinal tract. Sufficient work has been published, however, in regard to bacteriophages active against plant-pathogenic bacteria and root-nodule bacteria (Coons and Kotilla, 1925; Gerretson *et al.*, 1923-4) to make it evident that in the soil and in decaying vegetable accumulations fundamentally similar ecological relations exist.

It must be recognised first that nothing closely resembling the classical manifestations of bacteriophage lysis can occur in their natural habitat. It is obvious that if the same massive bacterial destruction and phage multiplication occurred all phage-sensitive bacteria would soon be eliminated but such massive lysis is a highly artificial phenomenon. It can only occur when the sensitive bacteria are actively multiplying in a medium which offers no hindrance to the diffusion of the liberated phage particles or any obstruction to their specific attachment to fresh bacteria. Such conditions can never occur in nature. In the alimentary canal the sensitive bacteria are suspended in a complex semi-solid mixture of food residues, mucus and other colloidal material, and enormous numbers of other bacteria. In diseased conditions, *e.g.* bacillary dysentery, the sensitive pathogenic bacteria are largely within the tissues and the possibility of free bacteriophage contact with such bacteria is still more remote. Dresel and Lewis (1930) have recently described some experiments which show clearly the inability of bacteriophages to destroy bacteria in animal tissues. They used tissue cultures which were infected with a phage-sensitive strain of *B. coli* and subsequently received the homologous phage. The phage had no obvious influence on the bacteria either in reducing their numbers or in provoking the appearance of resistant variants. A somewhat similar observation can frequently be made in cases of infantile dysentery due to the highly phage-sensitive Flexner bacilli. One may isolate from the same fragment of mucopus a highly active phage and normal phage-susceptible Flexner bacilli.

As in all host-parasite relationships a form of dynamic equilibrium must be set up by which a fairly constant proportion of both species manage to survive. In many intestinal infections it seems undoubted that the presence of the pathogenic bacteria allows a relatively large degree of phage multiplication at their expense but it is unlikely that the phage plays any important part in ridding the body of the pathogenic form. It is significant that in no natural bacterial infection of laboratory animals has the therapeutic administration of bacteriophage been shown to have

any real influence on the course of the disease (Topley, Wilson and Lewis, 1925; Bronfenbrenner and Korb, 1925; Colvin, 1932 *b*).

In addition to the classical parasite-host relationship of phages to bacteria there is another relationship around which has centred most of the prolonged controversy as to the nature of bacteriophage. This concerns the ability of certain bacterial strains, so-called lysogenic bacteria, to liberate phage during their growth. This capacity is surprisingly widespread particularly amongst the intestinal bacilli such as the coliforms and the *Salmonellas*. In a series of about 130 stock laboratory strains of various *Salmonella* types Burnet (1932) found that at least 93 regularly produced phage capable of lysing in typical fashion one or more of three "indicator strains" chosen specially for their wide susceptibility to phage action. All of these cultures gave perfectly normal colonies and except in two instances the phage produced had no apparent action on the strain from which it was obtained. Similar observations have been made by other authors. The two classical instances are the strain coli L first described by Lisbonne and Carrère (1922) and further studied by Bordet and Renaux (1928) and the strain coli 88 of Gildemeister and Herzberg (1924). Both these coliform bacilli produced a phage which lysed Shiga dysentery bacilli.

Lysogenic strains of this type produce phage consistently despite all attempts to free them from this property by such means as repeated re-isolation or growth in appropriate antiphage serum. Even when variants of the strain are tested they still retain the lysogenic attribute. One lysogenic strain of *B. enteritidis* gave rise to a whole series of variants, smooth and rough, motile and non-motile, mucoid and pellicle-forming, but all gave the same phage when grown in broth culture, only differing in the average amount produced (Burnet and McKie, 1929). It is obvious that in such cultures as this every cell must contain some rudiment of the phage which is liberated in growing cultures. If the organismal nature of bacteriophages is true for those liberated from lysogenic cultures as well as for the rest, and some unpublished filtration experiments indicate that the phage released from the *enteritidis* strain mentioned above has a particle size commensurate with that of other phages, one is almost forced to postulate that each bacterium carries in intimate symbiosis one or more phage particles which multiply by binary fission *pari passu* with the bacterium. Further the multiplication of phage and bacterium must be so co-ordinated that each daughter individual regularly receives the phage. Where the bacterial component of the symbiosis is a spore-former it can be shown that the lysogenic attribute is retained by the spore and is resistant to any degree of heat which still leaves the spore viable (den Dooren de Jongh, 1931; Cowles, 1931). The increased resistance to heat of the phage held within the spore can probably be correlated with the relative dehydration of the spore. Vedder (1932) has shown that phage particles are much more resistant to heat inactivation when dry than when suspended in watery solutions. On the whole it is not surprising that most of those who have studied such lysogenic bacteria are in favour of the view that the liberated phage is not an autonomous micro-organism but some unit of the bacterial "chromatin," a vagrant gene freed from normal constraint.

An intensive study of the types of phage liberated from a series of lysogenic

strains of the same bacterial species, however, makes this conclusion inadmissible. The *Salmonella* species *B. enteritidis* Gaertner has been most extensively studied from this point of view (Burnet, 1932). Amongst thirty-four strains three completely distinct types of phage, A, B and D were found, and at least amongst the B and D phages there were minor differences between phages of different origin. Of twenty-four lysogenic strains fourteen gave rise to B phage, seven to D phage, two gave both A and B phages and one A and D. The same random type of distribution obtained with *Paratyphosus* A and B and *aertrycke* strains from all of which examples of the well-characterised A phage were obtained as well as some of the less distinctive B, D and N types. In other words with these species the nature of the phage produced was not determined by the nature of the organism from which it was obtained. The results paralleled quite closely what would be obtained in a parasite survey of the intestinal tract of a number of examples from each of say four fairly closely related mammals but not what would be obtained in a comparative study of their leucocytes or spermatozoa.

In the same investigation, however, it was found that another group of *Salmonellas*, the *suispestifer* type and its relatives, did show the existence of a common type of lysogenicity. All the available strains of "Paratyphosus C" for instance including examples isolated in Russia, South America and the East Indies produced similar amounts of serologically identical phage. If the symbiotic interpretation is correct this can be regarded as the extreme of a series of continuously varying degrees of intimacy of association. Each individual of the species in this case carries the potentiality to produce one particular type of phage only. According to the results of Smith and Jordan (1931) this state may also hold for the diphtheria bacilli, all of the strains they examined being lysogenic toward a sensitive indicator strain of the same species. The *enteritidis* and paratyphoid group show much more variable behaviour in regard to the type of phage produced but each lysogenic strain is consistent in its activity and shows no gross evidence of lysis. Less stable degrees of association between phage and bacterium are also found. The "nibbled colonies" of partially resistant bacteria are familiar to all who have worked with phage and were actually described and illustrated by Gildemeister (1916) before d'Herelle's work appeared. They represent phage-bacterium associations in which the phage is capable at times of developing lytic activity against the bacterium. As in so many other biological associations it is quite impossible to draw any sharp line between extreme parasitism as exemplified in phages which lyse bacteria so completely as to sterilise the culture permanently, and the perfect symbiosis which has been postulated for *B. paratyphosus* C where the lysogenic character can actually be used as one of the characteristics defining the species.

It is extremely probable that nearly all bacteriophages *persist* in nature in such symbiotic or semisymbiotic associations. When circumstances, for example infective disease due to a phage-susceptible organism, introduce a population of abnormally sensitive bacteria into the same environment there is active phage multiplication of the type familiar in the test-tube but it is unlikely that any of the phage so produced will survive after the infection is eliminated.

It is a point of considerable interest that highly specialised pathogenic bacteria tend to be more susceptible to typical lysis and less likely to form permanent symbioses with phages than the less specialised forms from which they have presumably been evolved. A discussion of the possible significance of this finding will be found elsewhere (Burnet, 1932).

This article is not concerned primarily with the significance or lack of significance of bacteriophages in determining the outcome of bacterial infections, but some mention must be made of the work now being carried on in India on the phages lysing the cholera vibrio. It is definitely too early to attempt to assess these results but from the reports available it seems possible that the artificial dissemination of active phages through a community exposed to infection prevents the occurrence of the expected outbreak of cholera (Morison, 1932). If this is so it becomes an obvious possibility that the epidemiology of cholera is in large degree determined by phage-vibrio interactions. The proof or disproof of this hypothesis will require several years of large-scale investigation in the course of which one may hope not only for some practical control of cholera epidemics but for a satisfactory elucidation of some of the problems involved in the bacteriophage-vibrio association.

VII. THE ALLEGED PRODUCTION OF BACTERIOPHAGE FROM NORMAL BACTERIA.

Those who have opposed the conception of the micro-organismal nature of phage have found the chief experimental basis for their opinion in the widely published assertion that normal bacteria can by suitable manipulations be caused to produce bacteriophage spontaneously. Amongst recent writers Hadley (1928) has been prominent in providing evidence of this type and in his review of the subject extensive references to earlier observations will be found. The usual technique adopted has been to filter an old broth culture of the organism through a bacteria-proof filter and add the filtrate to a young culture of the same organism, again filter after a suitable interval and continue the process until evidence of lysis appears. Others have used various proteolytic ferments to initiate the appearance of phage in the bacterial cultures.

If a method were available which would regularly induce the appearance of phage from all cultures of those bacterial species which are sensitive to known phages, it would provide adequate evidence to discredit the micro-organismal theory of bacteriophages. No author has ever claimed any such success and many careful workers including some such as Bronfenbrenner (1928, p. 392) who have strongly opposed d'Herelle's theory have completely failed to obtain phage by the methods which were successful in other hands. Sporadic successes even if these involve a relatively high proportion of cultures must be heavily discounted in the light of (1) the known high incidence of admittedly lysogenic strains in certain species and (2) the very considerable technical difficulty of avoiding occasional "contamination" by phage in any laboratory in which bacteriophages are being handled.

Despite the prominence given to this question in bacteriophage literature one feels justified, in view of the mass of direct positive evidence in favour of the bio-

logical autonomy of the bacteriophages, in regarding all the experimental evidence of "spontaneous origin" as representing either activation of symbiotic phages pre-existing in the bacterial culture or laboratory contamination by extrinsic phage. In the study of lysogenicity amongst the *Salmonellas* already referred to (Burnet, 1932) the proportion of phages detected by the very simple technique used, was considerably higher than the proportion of successes claimed by most of those who have apparently succeeded in provoking the spontaneous production of phage. The possibility of contamination by phages in the laboratory environment has been stressed by both d'Herelle and Asheshov *et al.* (1933) and direct evidence of the way phage can be spread in dust or droplets in the air has been given by Colvin (1932). Several authors, notably Putter and Vallen (1923) and Beard (1931), have shown that phage may persist in filter candles which have not been rigorously resterilised.

VIII. CONCLUSION.

It should be clear that while the point of view which has been adopted is generally similar to that sponsored by d'Herelle there are some important divergences which on the whole tend to bring the interpretation of bacteriophage phenomena more into line with general biological thought. Bacteriophages are regarded as a class of diverse micro-organisms whose common feature is ability to parasitise or live in symbiosis with bacteria. They show approximately the same range of size and many of the functional characteristics, in particular inability to multiply in the absence of living cells, of the viruses which parasitise or live in symbiosis with animals and higher plants. It seems legitimate to regard the viruses as divisible primarily into three great groups according as they attack animals, higher plants or bacteria. Whether there is any evolutionary relationship between the three groups must remain problematical, but within that of the bacteriophages there is clear evidence of all the characteristic attributes of biological evolution, wide diversity of size, functional activity and chemical (antigenic) structure, but with a clear tendency to segregation into distinct species within which only minor variations occur. The differentiation into species appears to be about as distinct as is the case amongst the bacteria and in regard to their "pathogenicity" there are many analogies between the two groups of organisms. In both we find some that are parasitic on only one species of host organism, others capable of attacking a wide range of species, and just as bacterial strains vary in virulence, some phages have an acutely destructive effect on the susceptible bacterium while others show all grades of diminishing intensity of lysis until we reach the permanent phage-bacterium symbiosis characteristic of certain bacterial species.

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FACTORS AFFECTING THE PATHOGENICITY OF CEREAL FOOT-ROT FUNGI

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CONTENTS.

	PAGE
I. Introduction	351
II. The effect of soil conditions upon biological antagonism	353
III. Interpretation of soil temperature and moisture infection experiments	354
IV. Conclusions	359
References	360

I. INTRODUCTION.

EXPERIMENTS with the cereal foot-rot fungi have been especially hampered by the difficulty of controlling in a satisfactory manner the soil environment. Effective control of the environment must necessarily be preceded by adequate recognition of the various factors concerned. Thus the importance of soil temperature was recognised at a comparatively early stage in experimental work with these fungi, and has been investigated by Dickson (1923), McKinney (1923), McKinney and Davis (1925), Sanford (1927), Simmonds (1928 *a*), Dufrénoy and Frémont (1931), Gäumann (1932), Henry (1932), Vanterpool and Truscott (1932), and by Tanja (1933). Experiments on the effect of soil moisture content are reported by Dickson (1923), McKinney (1923), McKinney and Davis (1925), Simmonds (1928 *a*), Russell, R. C. (1931), Vanterpool and Truscott (1932), and by Tanja (1933). The most significant recent change in outlook upon the cereal foot-rot problem, however, has probably been brought about by recognition of the importance of the biological factor, and attention is now being concentrated upon the influence of other members of the soil microflora—fungi, bacteria and actinomycetes—upon infection by the cereal foot-rot fungi.

The importance of the reaction of one living micro-organism upon another, not only in culture but also in the soil itself, is now well established. A review of the literature upon this subject was made by Fawcett (1931) in his presidential address to the 22nd Annual Meeting of the American Phytopathological Society in December, 1930. Since then, attention has been concentrated especially upon the antagonism of other soil micro-organisms to infection by soil-borne fungus pathogens. Amongst

others may be mentioned papers by Bamberg (1931) and by Johnson (1931) upon the antagonism of certain bacteria to infection of corn by *Ustilago zeae*, by Konishi (1931) upon the antagonism of other soil bacteria to the root nodule bacteria of legumes, by Endo (1931, 1932, 1933) upon the antagonism of a large number of different bacteria and fungi to the fungi *Hypochnus centrifugus*, *H. Sasakii* and *Sclerotium oryzae-sativae*, and by Weindling (1932) upon *Trichoderma lignorum* as a parasite of other soil fungi. Coming more especially to the relation of biological antagonism to infection by the cereal foot-rot fungi, the importance of this was first emphasised by the Canadian investigators. The rapid deterioration of inoculum of the foot-rot fungi when added to soil, to which attention was first drawn by Simmonds (1928 b), was attributed by Broadfoot (1931, 1933 a) to the operation of this factor. This suggestion was followed up by Sanford and Broadfoot (1931) and by Broadfoot (1933 b), who were able to show that infection of wheat seedlings by *Ophiobolus graminis* was completely suppressed by the antagonistic effect of a number of different fungi and bacteria, not only by the living cultures, but also in many cases by their filtrates as well. Henry (1931) found that the growth of *Helminthosporium sativum* upon sterilised soil in small flasks might be completely inhibited by the addition of very small amounts of unsterilised soil, or by simultaneous inoculation with a number of other fungi and bacteria, so that no infection resulted when wheat seeds were inoculated with the contents of the flasks in 5-in. pots. Bisby, James and Timmin (1933) showed that *Trichoderma lignorum* was able to suppress the virulence of *Fusarium culmorum* and *Helminthosporium sativum* in pot tests.

Although the importance of the microbiological factor in soil-borne fungus diseases is thus coming to be generally recognised (Sanford, 1933) the influence of this factor will probably turn out to be more far-reaching than would appear at present. Two aspects in particular, namely, the real nature of antagonism, and the interrelation of the microbiological factor with soil temperature and soil moisture, have as yet received scant attention.

The influence of soil temperature and soil moisture upon infection by the cereal foot-rot fungi has been studied by a number of investigators (*vide supra*). Until recently, however, it has not been possible to draw any general conclusions as to the influence of these factors upon infection. It might well have been expected that the effect of temperature upon the infection of wheat and oats, by the different foot-rot fungi in the seedling stage would be broadly similar, for the following reasons. The optimum temperature for the development of seedlings of both wheat and oats is now generally recognised to be in the neighbourhood of 12–16° C. The optimum temperature for the vegetative growth of all the cereal foot-rot fungi studied lies within the range 25–30° C. An increase of infection might therefore be expected to occur with rise of temperature to 25° C., since the higher temperatures both accelerate the growth of the fungal hyphae, and depress, in a physiological sense, the vigour of the host plant (Dickson, 1923). It was therefore difficult to understand why increase of infection with rise of temperature should not be the general rule with these fungi. But no such uniformity was reflected in the results of experiments with

the different foot-rot fungi. In some cases infection was accelerated, in others depressed, by rise of temperature from 12 to 25° C. In a few cases different workers working with the same fungus obtained different results. The influence of soil moisture upon infection also seemed to vary in an equally unaccountable manner.

The explanation of this puzzling situation was suggested by a paper from Henry (1932) on "The influence of soil temperature and soil sterilisation on the reaction of wheat seedlings to *Ophiobolus graminis*." Henry found that whereas in unsterilised soil the general trend of the infection curve was downwards with rise of temperature, in sterilised soil the reverse was the case. The decrease in infection with rise of temperature in the unsterilised soil was attributed by Henry to the effect of temperature upon the biological antagonism of the soil microflora, which was considered to be negligible at 12° C., but very much more active at 25° C. His conclusions at once suggest that the results of previous experiments represent not the true effect of temperature upon infection, but a combination of this with the action of temperature upon the biological antagonism of the soil microflora. Henry's conclusions were confirmed by the experiments of Garrett (1934), who found that when wheat seedlings were grown in a naturally sterile river sand under conditions practically excluding the biological factor, infection by *Ophiobolus graminis* increased steadily with rise of temperature to 24° C., the optimum for vegetative growth of the fungus in culture.

In the light of these facts an examination has been made of all the available published work on the effect of soil temperature and moisture content upon infection by the cereal foot-rot fungi. It will appear that numerous contradictions in the infection curves become reconcilable, and that broad general conclusions may be drawn as to the effect of soil temperature and moisture content upon infection, if due allowance be made for the operation of the factor of biological antagonism. The importance of this factor must fluctuate widely with experimental conditions, so it will be as well to preface examination of the infection curves by a brief discussion of biological antagonism as it appears to be affected by different soil conditions.

II. THE EFFECT OF SOIL CONDITIONS UPON BIOLOGICAL ANTAGONISM.

Biological antagonism, in common with other soil microbiological processes, must be affected by the sum total of the conditions making up the soil environment. Only the following, however, will be considered here: (1) kind of inoculum, (2) soil temperature, (3) soil moisture content, (4) type of soil, (5) degree of sterilisation, and subsequent treatment.

(1) *Kind of inoculum.* With all inocula, whether simple spore suspensions of the fungus or cultures on rich organic media, a resistance seems to be opposed to the growth of the pathogen mycelium by the other members of the soil microflora. This effect is very much more pronounced with cultures of the fungus on organic media, such as oats and barley kernels, or mixtures containing cornmeal, than with spore suspensions. The added organic matter must influence considerably the soil microflora, although no detailed studies have yet been made on this point. But it appears

very probable that the rapid decrease in viability of such inoculum when added to soil (Simmonds, (1928*b*), Broadfoot (1931, 1933*a*), Sanford and Broadfoot (1931)) is due either to actual bacterial decomposition of the fungus culture, or to its inactivation by toxic substances excreted by the other micro-organisms.

(2) *Soil temperatures.* Although no work has yet been done with the definite aim of determining the effect of temperature upon biological antagonism, it will appear from examination of the infection curves that such antagonism increases with rise of temperature to 30° C.

(3) *Soil moisture content.* The infection curves indicate that, other conditions being suitable, biological antagonism increases with soil moisture content over the range 30–80 per cent. saturation.

(4) *Type of soil.* Different soils vary widely in their natural microflora. The influence of soil type upon biological antagonism is demonstrated by the experiments of Moritz (1932) and of Garrett (1934). In general, antagonism is least pronounced in soils with low organic content and low bacterial numbers, and most in evidence in rich, fertile soils.

(5) *Degree of sterilisation, and subsequent treatment.* Practically all investigators have employed sterilised soil in their experiments. Since the soil was generally sterilised only with the idea of killing extraneous plant pathogens, however, partial sterilisation only was often employed. The work of E. J. Russell and his collaborators (1932) has shown how rapidly bacterial numbers may rise in partially sterilised soil, finally much exceeding their original values. Even where the sterilisation employed has been complete, the subsequent treatment of such soil, which is generally shovelled about on a potting floor, and watered with non-sterile water, must be such as to ensure its rapidly becoming non-sterile once more. Of importance, also, must be the periods of time allowed to elapse between sterilisation of the soil, inoculation, and planting of the seed, respectively.

III. INTERPRETATION OF SOIL TEMPERATURE AND MOISTURE INFECTION EXPERIMENTS.

The experiments on the influence of soil temperature and moisture on infection by the cereal foot-rot fungi may now be examined. Several apparent anomalies become explicable if it be postulated that the true temperature-infection curve for all these fungi is a rising one with rising temperature, but that certain of them are very susceptible to biological antagonism from members of the soil microflora—a factor which comes increasingly into play at the higher temperatures. It is not desired to exclude altogether particular points of variation for certain fungi—one such is discussed in the case of *Gibberella saubinetii*—but it is considered that when the factor of biological antagonism is taken into account as well as soil temperature and moisture, the variability in the pathogenicity of the cereal foot-rot fungi becomes much more readily intelligible.

Taking first those experiments (grouped in Fig. 1) in which the fungus inoculum contained appreciable amounts of organic matter, being usually oat-barley kernel

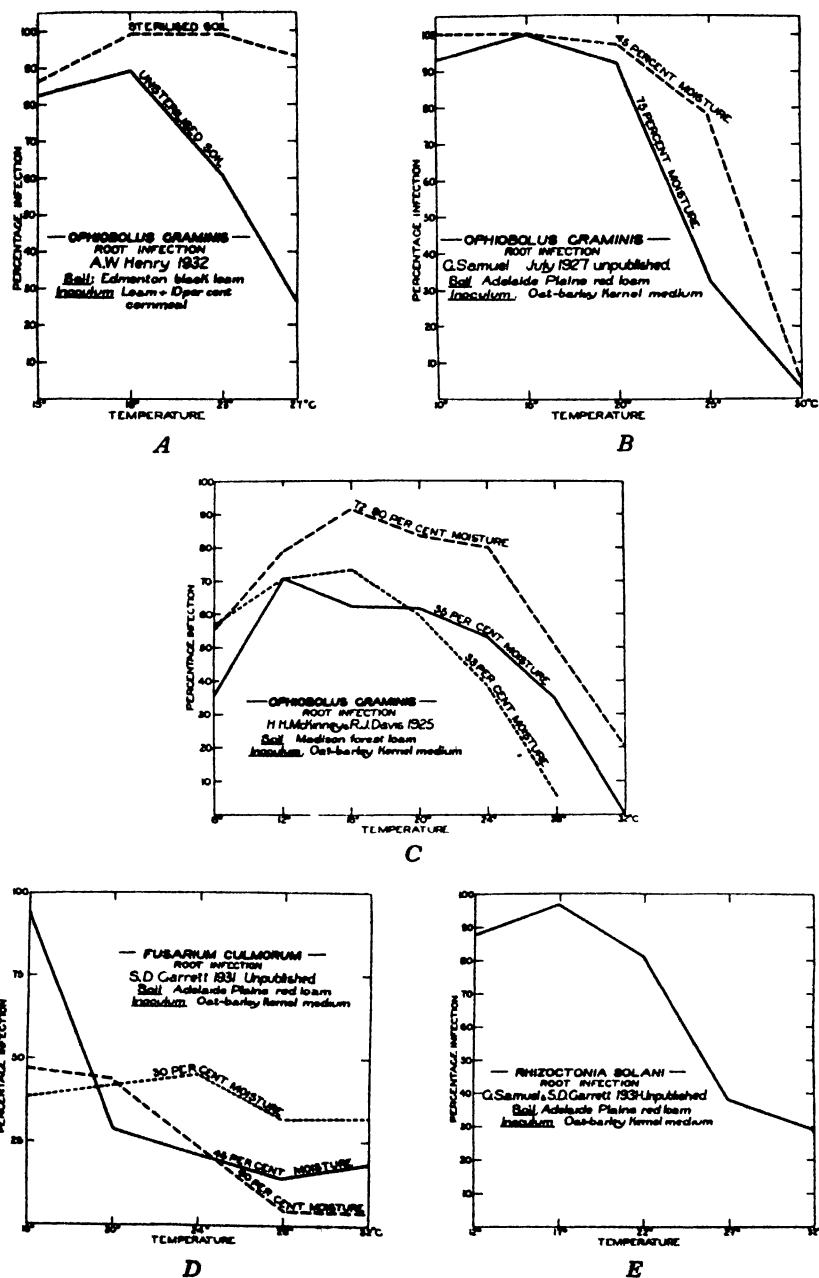


Fig. 1. Temperature-infection curves for cereal foot-rot fungi from experiments using cultures on organic media as inoculum. Downward trend attributed to increase of biological antagonism at the higher temperatures.

medium or a cornmeal mixture, it is seen that infection falls off with rise of temperature in every case (except in the case of *Pythium arrhenomanes* var. *canadensis*, which is discussed below). Some of the curves show, however, a preliminary rise to an inflection point in the neighbourhood of 16–18° C. This preliminary rise may correspond to the early part of the true temperature-infection curve, before deflection by the biological antagonism factor occurs.

In contrast to these are the experiments (grouped in Fig. 2) in which a spore suspension inoculum was employed and the addition of extraneous organic matter to the soil thus avoided. The infection curves show, in general, a rising trend with rising soil temperature. Apparently biological antagonism has not been marked under these conditions, and the curves probably reflect the true effect of temperature upon infection. In most cases, infection increased with rise of temperature to a point corresponding approximately to the optimum for growth of the fungus on culture media. Thus in the experiments of McKinney with *Helminthosporium sativum* (Fig. 2, A), the optimum temperature for infection is exactly the same as that for the vegetative development of the parasite, viz. 28° C. It is hard to resist the conclusion from these two groups of curves that excess organic matter in the inoculum favours the development of foreign micro-organisms, which have an antagonistic effect on the cereal foot-rot pathogens.

Vanterpool and Truscott (1932), working with *Pythium arrhenomanes* var. *canadensis*, the cause of Browning Root-rot of cereals in Canada, employed oat-barley kernel inoculum in two soil temperature experiments with this fungus. The results of the two experiments did not entirely agree, but in each case infection was higher at 31° C. than at 12° C., suggesting that biological antagonism had not increased sufficiently with rise of temperature to cause a decrease in infection. It is possible that *P. arrhenomanes* var. *canadensis* may be less susceptible to biological antagonism than other cereal root-rot fungi. It is certainly suggestive in this connection that browning root-rot differs from other cereal root-rots in that it is reported by these authors to be actually more serious after a summer fallow than after a preceding cereal crop. For it is becoming increasingly probable that it is biological antagonism and not starvation in the absence of a host plant that is responsible for the disappearance of inoculum of foot-rot fungi from fallows (Sanford, 1933).

Soil moisture content as well as soil temperature appears to influence biological antagonism. In two cases (Fig. 1, B and D) infection was found to fall off with rise in soil moisture content. R. C. Russell (1931), using oat-hull inoculum, also reported a decrease in infection with rise in soil moisture content for *Ophiobolus graminis*. This effect may tentatively be attributed to an acceleration of biological antagonism at the higher moisture contents. But in the experiments of McKinney and Davis with *O. graminis* (Fig. 1, C), exactly the opposite obtains, a fact which is hard to explain.

In the experiments in which spore suspension inoculum was used and biological antagonism is considered to have been reduced to a minimum, the true effect of soil moisture upon infection is to be sought. Infection increased with rise of soil moisture in the experiments of McKinney with *Helminthosporium sativum* (Fig. 2, A), and in

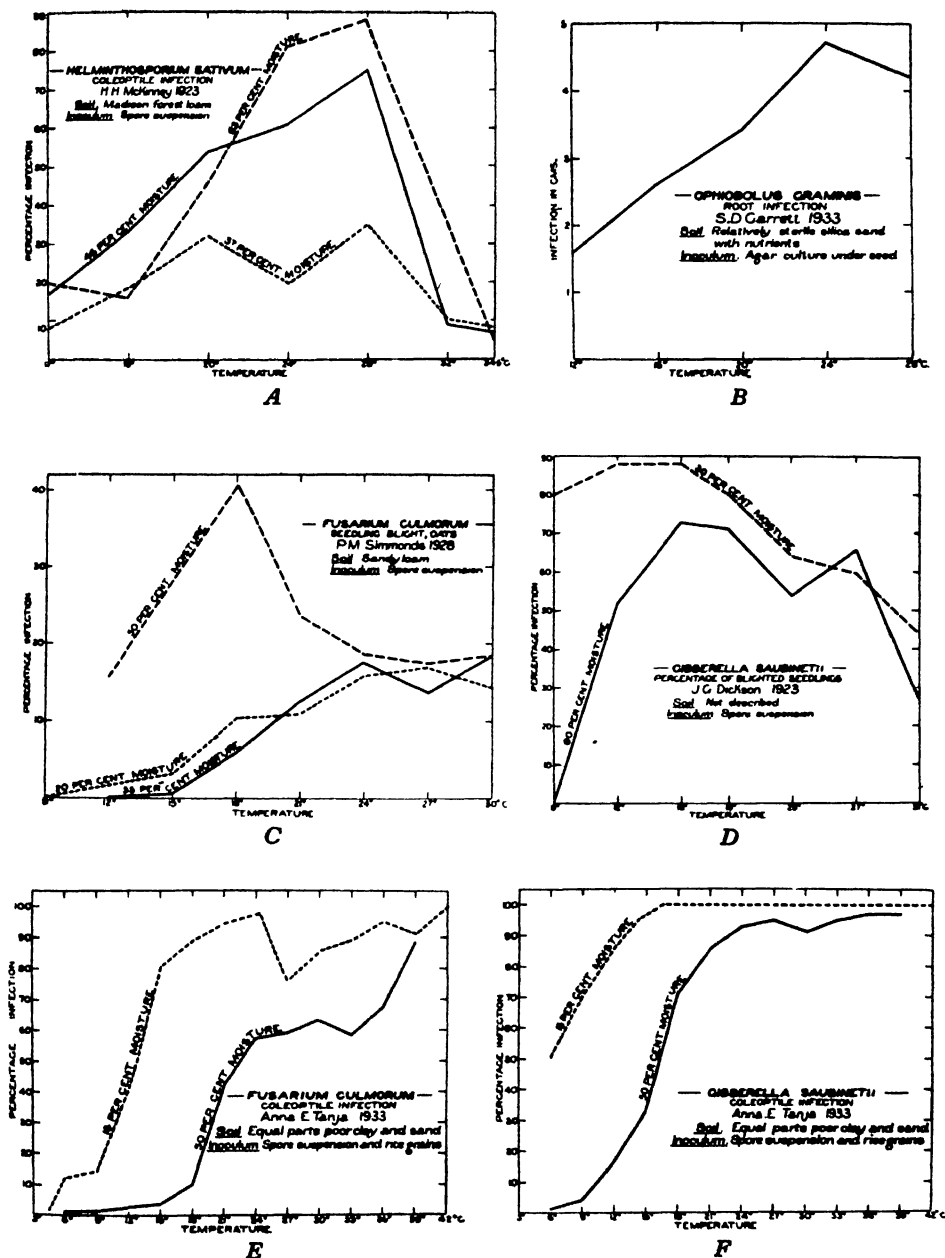


Fig. 2. Temperature-infection curves for cereal foot-rot fungi from experiments using spore suspensions as inoculum. Upward trend considered to represent the true effect of temperature upon infection.

those of Simmonds with *Fusarium culmorum* (Fig. 2, C). Such increase in infection with rise of soil moisture may reasonably be attributed, in part at any rate, to more favourable conditions for the activity of the fungus. But in the experiments of Dickson with *Gibberella saubinetii* (Fig. 2, D) and of Tanja with *G. saubinetii* (Fig. 2, F) and *Fusarium culmorum* (Fig. 2, E), infection increased with decrease of soil moisture content, although the general form of the temperature-infection curves indicated that biological antagonism could not have been responsible for this.

In the case of *Gibberella saubinetii*, Dickson and his co-workers (Dickson, Eckerson and Link, 1923) have shown that increase of infection at low soil moistures is to be attributed to an actual breakdown in the resistance of the seedling roots. In view of the exceedingly low soil moisture (15 per cent.) employed by Tanja in her experiments with *G. saubinetii* and *Fusarium culmorum*, it does not seem unreasonable to suggest that the resistance of the seedling roots to *F. culmorum* as well was altered by the exceptionally rigorous conditions.

It would appear from Dickson's work that the infection of wheat seedlings by *Gibberella saubinetii* is rather different from that by *Ophiobolus graminis* or *Helminthosporium sativum*, in that under favourable conditions, e.g. medium to high moisture, good light and temperatures below 12° C., the cell walls of the seminal roots develop a real resistance to this fungus. Thus Dickson (1923) remarks (p. 854) "a histological study of the wheat seedlings at the low temperatures demonstrated an abundance of the mycelium of the parasite around the subterranean portions of the seedlings, yet no penetration could be detected." (This fact appears on the infection curve for 60 per cent. moisture, which shows a fall to infection *nil* at 8° C.) Dickson suggested, therefore, that the great increase of infection under low soil moisture conditions at the lower temperatures was due to retardation of the development of this low temperature resistance. He found, moreover, that its development could also be inhibited by unfavourable light conditions, and that infection differences could be correlated with clear-cut biochemical differences in the composition of the roots (Dickson, Eckerson and Link, 1923).

Finally, the recent experiments of Tanja (1933) may be discussed in rather more detail. This work was done with two strains of *Gibberella saubinetii* and one of *Fusarium culmorum*. The inoculum employed was a heavy suspension of conidia obtained from a culture of the pathogen on rice. The rice grains were added to the soil as well, but this does not seem to have had the effect of promoting biological antagonism to any appreciable extent, for the temperature-infection curves for both fungi rise steadily to 30° C. at both soil moistures employed (15 and 50 per cent. saturation). These curves are exceptional in one particular, in that they show no decline in infection above 30° C. Indeed, Tanja explicitly concludes (p. 424) that "These results agree neither with those which Gäumann had tabulated according to Jones, nor with those of Dickson. Both these authors came to the conclusion that there was a decrease in the attacks at temperatures exceeding 30° C." (Reference has already been made to the work of Dickson (1923); the citation of Jones (1924) refers to a review paper by this author on the relation of environment to plant disease.)

In Tanja's work two sources of error, one in technique and the other in expres-

sion of data, may have contributed to the results obtained. In the first place, no precautions were taken to ensure that the temperature of the surface soil was the same as that of the water in the tanks. Tanja admits that at 42° C., the difference between the temperature of the tank water and that of the soil at seed depth (3 cm.) was as much as 7° C., but states (p. 406): "For practical reasons, we preferred to hold the water in the tanks as constant as possible at the desired temperatures, and to take only those into consideration, instead of the temperatures which in actual fact prevailed at seed-depth." This discrepancy would not have entailed serious error in the estimation of root infection, since the root system of the plants is generally below the water level in the tanks. But in the experiments under discussion, infection figures were derived from coleoptile infection, which would occur just in that upper layer of soil over which the temperature gradient must have been steepest.

Secondly, the infection figures from which the summary curves are drawn represent the percentage of plants above ground infected by the fungus. No allowance was made for plants killed below ground, though these reached a percentage of 70 in some experiments. The deaths below ground attributable to infection by the fungus do indeed show a sharp decline as soon as temperatures exceeding 25-30° C. are reached, in practically every case.

IV. CONCLUSIONS.

The experiments discussed above show that the effect of soil temperature and moisture content upon infection under sterile conditions may be actually reversed in the soil, owing to the operation of the microbiological factor. The effect of soil temperature upon infection by a fungus pathogen has previously been ascribed to its combined influence upon the vigour of the fungus and upon the resistance of the host. But it is now evident that the effect of other soil conditions, and particularly that of the microbiological equilibrium, upon the fungus may vary markedly at different temperatures.

Soil microbiologists have shown that soils vary widely in their natural microflora. The experiments of Moritz (1932) and of Garrett (1934) suggest that such differences in the microflora of different soils may account for differences in the relative prevalence of the take-all disease of wheat (caused by the fungus *Ophiobolus graminis*) in different districts. The poorer the natural microflora of any soil, the more likely is the influence of soil temperature and moisture content upon infection in that soil to correspond to that obtained in experiments with the pathogen in pure culture.

It seems very likely, however, that the different foot-rot fungi may vary considerably in their susceptibility to biological antagonism. Variations in the physiological state of the mycelium under different soil conditions may be still more important. The dark-coloured resistant "runner" mycelium, by means of which certain fungi spread through an inhospitable soil, must differ physiologically as well as morphologically from the colourless, thin-walled protoplasmic mycelium generally formed upon an organic substrate, which is probably particularly sus-

ceptible to biological antagonism. (The influence of soil type upon biological antagonism may thus operate directly through the resistance of the pathogen as well as through the antagonistic organisms.)

Enough has been said to indicate the complexity of the problems still awaiting solution in this field. It is possible that these considerations may eventually throw some light upon one of the more interesting questions still awaiting solution—that of the distribution of the cereal foot-rot pathogens in wheat-growing countries. Thus, taking an example from Australia, experience has shown that in New South Wales the most important root-rot pathogen of wheat is *Helminthosporium sativum*. In the State of South Australia, on the other hand, almost the whole of the damage due to root-rot is caused by *Ophiobolus graminis*, the take-all fungus. Extensive isolation work has shown that *Helminthosporium sativum* is widely distributed throughout South Australian soils, so that its failure to act as a serious pathogen in this State is still in need of explanation.

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THE DIGESTION OF WOOD BY INSECTS AND THE SUPPOSED ROLE OF MICRO-ORGANISMS

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CONTENTS.

	PAGE
I. Historical	363
II. The occurrence of intracellular micro-organisms in wood-eating insects and the supposed role of such micro-organisms	366
III. The occurrence of extracellular intestinal micro-organisms in some wood-eating insects	368
IV. Experimental work on the digestion of different components of wood by insects	371
1. The components of wood	371
2. The fate of the wood components in xylophagous insects	373
(a) Cellulose-splitting enzymes	373
(b) Hemicellulose-splitting enzymes	375
(c) Enzymes splitting starch, sugars and proteins	376
(d) Discussion	378
V. Summary	379
References	380

I. HISTORICAL.

LYONET is to be credited with being the first author to carry out experimental work in connection with the feeding habits and the nutrition of wood-eating insects. In 1762, being struck by the ability of *Cossus ligniperda* to live and develop on a diet of dry wood, he conducted an interesting anatomical and physiological research on the larva of this species. He was unable to observe a dissolution of wood particles through the action of any of the juices of the alimentary canal or its related glands. Having observed, however, that the salivary glands in these larvae are exceptionally large in size, he referred to them as the "dissolving means" though he was unable to notice any apparent change in the condition of the wood after being exposed to the action of the contents of these glands.

In 1875 the same species of *Cossus* was the subject of investigation by Plateau. According to this author there is no amylase either in the secretions from the salivary glands or from the digestive epithelium. Unlike what was known to occur in other insects, starch was left quite intact when exposed to the action of these secretions. Nevertheless, Plateau considers the sugar present in the contents of the mid-gut to be a product of digestion.

The absence of cellulase and amylase from the contents of the salivary glands of *Cossus ligniperda* was also demonstrated by Henseval (1895, 1897). According to this author, the secretion has no effect whatsoever on particles of wood, and he concludes

therefore that the secretion of these glands has probably only an antiseptic effect against micro-organisms.

The line of research followed by these three investigators indicates the first attempts towards the elucidation of the methods by which insects can get their food from wood, and shows the trend of physiological research on this subject up to the end of the nineteenth century.

In the first year of the present century, however, a new factor was introduced into the field of physiological research on insect nutrition. In that year Escherich (1900), after a brief study of the intracellular micro-organisms occurring in *Sitodrepa panicea* (*Anobium paniceum*), advanced the view, previously hinted at by Karawaiew (1899), that the intracellular micro-organisms associated with the alimentary tract of certain insects play a vital role in the digestive processes of their host. This view is probably inspired by the hypothesis originally put forward by Krassiltschik (1889) that a symbiotic relationship exists between intracellular micro-organisms and their insect hosts.

As far as we have been able to ascertain, the first suggestion that micro-organisms play a vital role in the digestive processes of *wood-eating* insects, owes its origin to Portier (1911). Although the work of this author was unfavourably reviewed (Lumière, 1919), nevertheless his idea of the symbiosis between micro-organisms and wood-eating insects outlived all criticism. It is mainly due to the work of Buchner and his school that this view has become established and is now accepted by almost all workers on the nutrition of wood-eating insects (Jordan, 1913; Oppenheimer, 1925; Yonge, 1926; Campbell, 1929; Uvarov, 1929; Pringsheim, 1932).

Through the activity of Buchner we now know of the presence of intracellular micro-organisms in a large number of insect families. Buchner refers to such micro-organisms as symbionts, playing a vital part in the life of their hosts. In the case of insects with wood-eating habits, Buchner attributes the breaking down of cellulose for use by the insect host to the symbionts which it harbours. A co-ordinated study in Buchner's laboratories led to the discovery of micro-organisms or symbionts in representatives of a number of insect families. Such association has been found to be of rather frequent occurrence among those insects with wood-eating habits. Buchner considers this fact to be of great importance in supporting his theory regarding the symbiotic relationship between wood-eating insects and micro-organisms. The assumptions current among biologists that wood is exceedingly poor in nutritive substances and that wood-eating insects are unable to secrete a cellulase, are also taken by Buchner as lending valuable support to the view he maintains. This author also quotes the results of Werner (1926 *a, b*) in support of his view. According to Werner, the larvae of *Potosia*, which live in the nests of *Formica rufa*, harbour intestinal bacteria in well-developed pouches in the proctodaeal region. These bacteria have been identified as *Bacillus cellulosa fermentans* and their ability to break down cellulose has been experimentally demonstrated. The work of Cleveland (1924-8) on termites with intestinal Protozoa has also helped a great deal in establishing the view under consideration.

Notwithstanding the amount of work done in the elaboration of Buchner's view

and notwithstanding the encouraging reception it has been given by biologists, Uvarov, though inclined to accept the theory of symbiosis, writes (1929, p. 266) that "... wood-eating insects must be classified as insects of unknown feeding habits. This is an astonishing conclusion at which to arrive with regard to a group of insects of great economic importance and one which is very widely spread and of common occurrence."

In 1930, however, Mansour showed that during the larval stage of *Hylobius abietis*, when the insect's food consists entirely of wood, the micro-organisms remain outside the muscular layer of the alimentary canal of their host, without any access to the lumen. But during the imaginal life, when the food is no longer wood, the micro-organisms, having changed their relative position during metamorphosis, pass in large numbers into the lumen of the gut and mix with the food therein. The view of symbiosis between wood-eating insects and micro-organisms appeared, therefore, to be based on insufficient evidence.

In 1930 Ripper showed that a number of wood-eating insects depend entirely on their own digestive secretions (independent of the occurrence of micro-organisms) in deriving the necessary nutriment from the wood they live on. Ripper thus cast doubt on Buchner's view from the physiological side.

In 1930 also Wiedemann gave an account of the relationship between some lamellicorn larvae and their intestinal micro-organisms. The interpretation of this author is totally different from that given by Werner (1926 a). According to Wiedemann, the intestinal micro-organisms' of the lamellicorn larvae are utilised as a direct food source.

Recently Mansour (1934 a, b) published work on the intracellular micro-organisms occurring in certain beetles. He firstly points out that the intracellular micro-organisms occurring in two wood-eating bostrychid species are separated from the alimentary tract throughout the larval and imaginal life of their host. Secondly, he draws attention to the fact that evanescent and empty mycetomes do occur. Thirdly, he shows that in *Baris granulipennis*, an atrophic curculionid, intracellular micro-organisms are present, but that they leave the host very soon after its emergence from the pupal stage, and that the imaginal life, which lasts for a number of months, is continued in spite of the absence of the micro-organisms. These three points are considered by the author to furnish conclusive evidence for the lack of a necessary relationship between the occurrence of micro-organisms and the feeding habits of their host.

Mansour and Mansour-Bek (1933-4) carried out physiological studies on the digestion of wood by insects. True wood-eating insects, independent of micro-organisms, are of two categories: one secretes a cellulase and can therefore live on kinds of wood with a very low starch and sugar content, while the other does not secrete cellulase, and is therefore confined to species of wood with a relatively high sugar and starch content. These authors, in the light of their own results and those of Wiedemann (1930), give a new interpretation to the observations of Cleveland (1924-8) on the termites with intestinal Protozoa: the flagellates of the termites may be utilised as a direct and supplementary food source.

Wilson, in 1933, published an account of a biological and ecological study on *Lyctus*, claiming that this beetle only infests timber in which starch is prevalent, and that the beetle and its larva live entirely on starch.

II. THE OCCURRENCE OF INTRACELLULAR MICRO-ORGANISMS IN WOOD-EATING INSECTS AND THE SUPPOSED ROLE OF SUCH MICRO-ORGANISMS.

It is mainly through the researches of Buchner and his school (1927-33) that we know now of the existence of intracellular micro-organisms in numerous wood-eating insects. The known cases may be grouped as follows:

(1) Insects with intracellular micro-organisms in connection with the alimentary canal in both larval and adult stages, *e.g.* some Anobiidae (Buchner, 1921; Breitsprecher, 1928) and some Cerambycidae (Heitz, 1927) among the beetles.

(2) Insects having intracellular micro-organisms without access to the alimentary canal in the larval stage, the organisms passing freely into the lumen of the gut during imaginal life, *e.g.* some Curculionidae (Buchner, 1928, 1933; Mansour, 1930; Scheinert, 1933).

(3) Insects with intracellular micro-organisms away from the alimentary canal, *e.g.* some Lyctidae (Gambetta, 1928) and some Bostrychidae (Mansour, 1934*a*).

In all the cases where intracellular micro-organisms occur, the mode of transmission from one generation of the host to the next insures the infection of all the eggs. The infection takes place at different stages of the egg in the different families. Four methods of transmission have been described.

The first method of transmission was originally described by Buchner (1921) and later confirmed by Breitsprecher (1928). According to these authors, in some Anobiidae, where yeast-like micro-organisms occur in four pouches at the anterior end of the mesenteron, the micro-organisms pass through the anus and infect the female genital apparatus, and this starts a culture in certain sacs, which were called by Buchner (1921) the intersegmental tubes of the smearing apparatus. During egg-laying, a portion of the contents of these sacs is squeezed out and the yeast-like micro-organisms are thus spread on the surface of the egg shell. During hatching the issuing larva ingests a piece of the shell and thus the micro-organisms are introduced into the alimentary canal.

The second method of transmission was described by Mansour (1930) in *Calandra oryzae* and *Hylobius abietis*, and confirmed by Buchner (1933) and Scheinert (1933). Infection is here accomplished in the oocyte stage. The female genital rudiment is intimately associated with mycetocytes, which in the imago occupy the anterior tips of the ovarioles. During oogenesis the micro-organisms break loose from these mycetocytes, mix with the nurse cells and finally invade the young oocytes long before the deposition of yolk or the appearance of the follicular cells. The micro-organisms thus introduced are first scattered between the yolk globules. During embryonic development they invade some of the cells proliferated from the mesenteric rudiments and form the alimentary bacterial cell mass which exists in

both males and females. In the females another bacterial centre is formed in close association with the genital rudiment.

The description of the third method we owe to Gambetta (1928) and Koch (1931). In the Lyctidae and Cucujidae infection takes place through the invasion of the female genitalia by micro-organisms which break loose from the mycetomes. The latter lie in the body cavity independent of all other systems. The infection of the eggs takes place after the appearance of follicular cells.

The fourth method occurs in some bostrychid beetles (Mansour 1934*a*). Here infection takes place through the male genitalia. The testis lobes are invaded by micro-organisms breaking loose from the mycetomes. This invasion leads to a great disturbance of the process of spermatogenesis, especially in aged males, and ultimately the micro-organisms mix with the sperms. During copulation they are passed with the sperms into the female genitalia, where they remain in the bursa copulatrix and hence enter into the fully formed egg through the micropyle.

The regular occurrence of intracellular micro-organisms in all individuals of one species, the elaborate methods of transmission, and the close association of some micro-organisms with the alimentary tract of their host, are taken by Buchner as an indication of a symbiotic relationship between micro-organisms and host, the former breaking down cellulose for use of the latter. But while it is possible that micro-organisms having access to the alimentary canal may have such a role, those which never have access to the digestive epithelium or the lumen of the gut of their host could not play this part.

Biological and morphological studies. In his study of the intracellular micro-organisms of *Calandra oryzae* and *Hylobius abietis*, Mansour (1930) points out that the cells harbouring intracellular micro-organisms in the larval stages of these two species are situated outside the muscular investment of the alimentary canal and have no means of access to the lumen of the gut or to the digestive epithelium. During metamorphosis, however, the mycetome surrounds the developing mid-gut tube and the mycetocytes ultimately become localised around the cores of the mesenteric caeca. During the imaginal life of these two species, the micro-organisms pass from their harbouring cells and mix with the gut contents. Larvae and adults of *Calandra oryzae* live on similar food materials, which are very rich in starch and ordinary food ingredients. In the larvae the micro-organisms do not mix with the gut contents, while in the adult stage they pass in great quantities from their harbouring cells and mix with the food. It appears, therefore, that these micro-organisms do not play any important part in the digestive processes of the host.

In the case of *Hylobius abietis*, the larvae actually live on wood. The micro-organisms at this stage have no access to the lumen of the gut. The adult, on the other hand, is not a wood-eater, but the micro-organisms have been observed to pass into the lumen of the gut in large quantities.

Buchner (1933) records the presence of mycetomes similar to those of *Calandra oryzae* and *Hylobius abietis* in no fewer than seventy-four species of curculionids, referring to the micro-organisms as symbionts. Scheinert (1933), one of Buchner's

students, published an account of the intracellular micro-organisms in *Hylobius abietis* and other weevils, but did not discuss the question of symbiosis.

Mansour (1934*b*) showed that there exists in *Calandra granaria* a mass of cells with a developmental history similar to that of the mycetome of *C. oryzae*, but quite sterile. *Calandra granaria* may thus be looked upon as a deflorated *C. oryzae*. This view gains support from the work of Liliensern (1932), who records the occurrence of sterile mycetomes in two species of ants. She also points out the remarkable fact of the natural occurrence of sterile mycetomes in some individuals of certain infected species. Moreover, she records the regular occurrence of sterile mycetomes in a variety of an infected species (*Formica fusca* var. *glebaria*).

These facts concerning the occurrence of sterile mycetomes are considered by Mansour (1934*b*) to preclude any idea of symbiosis between such intracellular micro-organisms and their hosts. He also quotes, as evidence against the views of Buchner, the case of the atrophic weevil, *Baris granulipennis*, where the intracellular micro-organisms, in association with the alimentary canal, all pass to the outside soon after emergence, and the case of some wood-eating Bostrychidae and Lyctidae, where intracellular micro-organisms occur but are always away from the alimentary tract.

Experimental observations. Heitz (1927) deals mainly with the intracellular micro-organisms of cerambycid and anobiid wood-eating species. He succeeded in cultivating the micro-organisms on artificial media. The wood-eating species studied were *Rhagium* spp., *Leptura rubra* and *Spondylis buprestoides* (fam. Cerambycidae), and *Ernobius abietis* (fam. Anobiidae). The micro-organisms of these species grew only on liquid or agar media of bouillon, sausages or glucose. The growth was quite intensive in the case of the micro-organisms of *Ernobius* and *Leptura*. This author admits that he was quite unable to procure direct evidence to support the view that these micro-organisms break down cellulose. It is also remarkable that the growth behaviour of the micro-organisms of these wood-eating species was found to be similar to that of the micro-organisms of *Sitodrepa panicea*, which does not live on wood.

Koch (1931, 1933) has been occupied with the process of defloration. In 1931, during his studies on *Oryzaephilus surinamensis*, a pest of stored food materials, which possesses four big mycetomes, he was able to get normal beetles with mycetomes quite free from micro-organisms. Koch (1933) claims, from his defloration and feeding experiments on *Sitodrepa panicea* (not wood-eating), that the intracellular yeast-like micro-organisms are utilised as a source of vitamins.

III. THE OCCURRENCE OF EXTRACELLULAR INTESTINAL MICRO-ORGANISMS IN SOME WOOD-EATING INSECTS.

Most of the recent work on this subject we owe to Kofoed and Swezy (1919) and Cleveland (1924-8) on the Protozoa-harboursing termites, Werner (1926*a, b*) and Wiedemann (1930) on the presence of micro-organisms in the proctodaeal pouch of some lamellicorn larvæ, and Buchner (1930) on the occurrence of micro-organisms in the proctodaeal caecum of some Tipulid larvae.

The occurrence of micro-organisms in well-developed regions of the alimentary canal of some wood-eating insects has often been referred to as evidence for the symbiotic relationship between the micro-organisms and their hosts. According to this view the micro-organisms break down cellulose for use by their host. The work of Werner (1926 *a, b*) and of Cleveland (1924-8) has often been quoted as proof of the validity of this conclusion.

The larvae of *Potosia cuprea*, which are found in the nests of *Formica rufa* and also in rotting wood stumps, have been found by Werner to harbour a flourishing fauna, some members of which have been studied and their ability to break down cellulose ascertained. Werner concludes that there is a symbiotic relationship.

However, the more thorough investigation on the feeding habits of *Oryctes nasicornis*, *Osmoderma eremita* and *Cetonia marmorata* carried out by Wiedemann (1930) points to quite a different explanation for the presence of micro-organisms in the alimentary canal of such insects. The larvae of the species referred to above also live in rotting wood and possess proctodaeal chambers similar to those of *Potosia*. These chambers similarly contain a rich gathering of micro-organisms, mainly bacteria and flagellates. These are not restricted to the chambers of the hind-gut, but are also scattered thinly in the mid-gut region. As in the case of *Potosia*, the micro-organisms are first introduced into the alimentary tract with the rotting wood ingested by the larvae. In the proctodaeal chambers they find favourable life conditions and there they flourish.

Wiedemann showed experimentally that some of the bacteria present are able to cause a splitting of cellulose. Owing to the presence of a very rich companion flora which was found unable to attack this substance, it appeared to this author that all the products of cellulose breakdown are utilised by the micro-flora. The flagellates present were found to be unable to live on wood. They were seen to ingest bacteria, but the bacteria-flagellate ratio remained constant.

The micro-organism complex referred to above grows best and is thickest in the alkaline or neutral regions of the alimentary tract. The acidity of the gut was found to increase gradually from the posterior region of the mid-gut backwards. The latter region was found to be distinctly alkaline, while the hind region of the proctodaeal chamber and the part posterior to it gave acid reactions. The alkalinity of the mid-gut is due to the nature of its secretions, while the acidity of the posterior region is attributed to the activity of the bacteria.

The mid-gut epithelium secretes a protease which is active only in acid media. This protease therefore passes backwards quite inactive until it comes to the acid region of the gut. Here it digests the micro-organisms (bacteria and flagellates) for assimilation by the host larva. Wiedemann records the presence of half-digested flagellates in this acid region. This explains the comparative scarcity of the micro-organisms in the hind region of the proctodaeal chamber. The products of digestion are absorbed in special zones of the walls of the hind-gut.

Wiedemann (1930, p. 254) sums up the principal steps in the nutrition of the lamellicorn larvae he studied as follows: "... Micro-organisms and their spores or cysts are taken in with the food material. The mid-gut epithelium secretes an alkaline

fluid, which mixes with the food. . . . This secretion also contains a protease which is only active in an acid medium. The micro-organisms pass thus unaffected through the mid-gut and in the hind-gut they flourish under the excellent life conditions prevailing in the big pouch. . . . The bacterial association in the hind-gut can break down cellulose, and acids are formed. This hydrolysis takes place. . . especially in well-marked zones. . . . Through the activity of the bacteria the pH changes gradually, and once the reaction has become acid, the protease becomes active, and digests the bacteria and the flagellates. The absorption of the products of this digestion. . . takes place in special zones. . . " (*loc. cit.* p. 254). The lamellicorn larvae referred to must therefore be considered as micro-organism-feeding insects.

The observations of Cleveland (1924-8) on Protozoa-harbouring termites are also open to a similar interpretation. Cleveland (1925*a*) observed that the intestinal flagellates ingest solid pieces of wood. Consequently the digestion of wood by these flagellates must take place inside their body. According to Uvarov (1929), a similar conclusion has been arrived at by Buscaloni and Comes (1910). It is, therefore, difficult to understand how the termite can benefit directly from the wood through the activity of the Protozoa.

Cleveland (1925*d*) has pointed out the possibility of the digestion of the intestinal flagellates in the alimentary canal of their termite host. He writes (p. 315): "In nature, termite Protozoa may aid their host by giving themselves as food. It is not known how long the life-cycle of these Protozoa is, but if it is not longer than that of the parasitic Protozoa that have been cultivated in artificial media from a single individual, countless millions of them must die daily in a single termite."

This author also writes in 1928 (p. 232), in his discussion of his feeding experiments on defaunated termites: ". . . They show however that none of these termites are able to live indefinitely after their Protozoa have been removed by oxygen treatment. But in most cases they do not show why some termites lived longer than others. This may be a difference inherent in the termites themselves, some being more dependent on Protozoa than others. Further investigations in fact may even show that certain Protozoa-harbouring termites are able to live for a long time if not indefinitely on a normal diet of wood after their Protozoa have been removed." This, of course, indicates clearly, as already pointed out by Uvarov (1929), that Protozoa-harbouring termites are able to extract some nutritive substance from wood without the help of their Protozoa.

In view of the fact that termites can live for some time after the removal of their intestinal Protozoa, and also that these Protozoa ingest solid wood particles and that they are digested in the alimentary canal of their host, it may be concluded that the termites utilise their intestinal Protozoa as a direct and supplementary food source. Protozoa-harbouring termites are in this respect similar in their feeding habits to the lamellicorn larvae with intestinal micro-organisms.

IV. EXPERIMENTAL WORK ON THE DIGESTION OF DIFFERENT COMPONENTS OF WOOD BY INSECTS.

(1) THE COMPONENTS OF WOOD.

Reference has already been made to the supposed low content of ordinary nutritive substances, such as sugars and starch, in wood, and to the fact that this assumption forms one of the foundations of Buchner's view as to the symbiotic relationship between wood-eating insects and micro-organisms. Wilson (1933, p. 662) writes: "Indeed, wood has seemingly been regarded solely as ligno-cellulose while the cell-contents, except for water, have been conspicuously ignored."

Most wood analyses carried out in recent years show that the composition of the various kinds of wood is widely different. The greater part of wood is formed in all cases by cellulose, associated with lignin, thus forming the proper woody substance, ligno-cellulose. Whether the lignin is adsorbed to the cellulose, or in chemical combination with it, is still a subject of investigation by chemists.

Cellulose, which is the main substance of the cell wall, forms from 40 to 62 per cent. (dry weight) of the constituents of wood, while lignin is present in amounts varying from 18 to 38 per cent. of the dry weight (Schorger, 1926; Hawley and Wise, 1926).

An important group of constituents of wood is formed by the so-called hemicelluloses, a name introduced by Schulze (1892). Contrary to the insoluble cellulose, hemicelluloses are relatively insoluble in water, but soluble in alkalis and acids, and readily hydrolysed by the latter into simple sugars. This group, which is not very well defined, comprises polysaccharides, built up largely of anhydropentose- and anhydrohexose-residues, which can be represented by the formulae $(C_5H_8O_4)_n$ and $(C_6H_{10}O_5)_n$ and in intermediate cases, such as xylo-mannans, etc., by formulae intermediate between these two. These hemicelluloses may function in part as polysaccharides of the cell wall, in part as reserve material in the cell. The structural hemicelluloses present in the cell wall consist chiefly of pentosans and yield on hydrolysis pentoses, e.g. xylose and arabinose, while most of the hexosans are present

Table I. *Hemicellulose content of different kinds of wood.*

Wood	Pentosan	Hexosan	Hemi-cellulose total	Author
Western yellow pine (<i>Pinus ponderosa</i>)	7.35	7.15	14.6	Ritter and Fleck (1922), Dore (1920a)
Pine (<i>Pinus silvestris</i>)	11.02	12.78	25.8	Schwalbe and Becker (1919), König and Becker (1919)
Fir (<i>Picea excelsa</i>)	8.67	13.58	22.25	König and Becker (1919)
Oak (<i>Quercus densiflora</i>)	19.59	1.56	21.15	Ritter and Fleck (1922), Dore (1920b)
Birch (<i>Betula verrucosa</i>)	27.07	4.61	31.68	Schwalbe and Becker (1919), König and Becker (1919)
Poplar (<i>Populus tremula</i>)	23.75	2.60	26.35	" "
Beech (<i>Fagus silvatica</i>)	24.86	4.36	29.22	" "
Ash (<i>Fraxinus spec.</i>)	19.29	5.70	24.99	König and Becker (1919)
Willow (<i>Salix spec.</i>)	16.75	5.05	21.8	" "

more often in the form of reserve material. The amount of pentosans in different kinds of wood varies from 6 to 23 per cent., that of hexosans from 2 to 14 per cent. (see Table I).

Other carbohydrates in the wood, the occurrence and quantities of which have not been studied as thoroughly as those mentioned above, are starch and soluble sugars (*e.g.* glucose and saccharose) present in the cell contents. The data available (see Table II) show that the starch content varies from 0 to 5.9 per cent., and the sugar content (calculated as glucose) from 0 to 6.2 per cent.

Table II. *Starch and sugar content of different kinds of wood.*

Wood	Starch	Author	Soluble sugar (in glucose)	Reducing sugars (in glucose)	Author
Maple	2.65	Beckmann (1915)	Outer base 1.45 Inner base 0.73	Incl. 0.71 Incl. 0.40	Jones (1916)
Birch	0.95	"	—	0.2	"
Beech	—	"	1.59	0	Ripper (1930)
Alder	1.54	"	—	—	Campbell and Taylor (1933)
Elm	5.90	"	—	—	
Sweet chestnut	2.65	"	—	—	
Poplar	—	"	—	2.27	Ripper (1930)
Mulberry	0.2	Mansour and Mansour-Bek (1934)	0.27	—	Mansour and Mansour-Bek (1934)
Poinciana	4.5	"	1.1	—	"
Tamarix	4.4	"	2.8	—	"
Albizzia sapwood	3.9	"	6.2	—	"
Albizzia heartwood	0.5	"	0.2	—	"
Juniperus	—	"	3.78	3.13	Campbell and Taylor (1933)

Substances present in wood, other than those discussed above, include the tannins, resins, dyes, essential oils, gums, alkaloids, waxes, fats and proteins. This last-mentioned group of substances, which is of vital importance as a nutritive substance, is present in amounts varying from 1.1 to 2.3 per cent. of the dry weight (see Table III).

Table III. *Protein content of different kinds of wood.*

Wood	Protein in % of dry weight	Author
Pine (<i>Pinus silvestris</i>)	1.27	König and Becker (1919)
Fir (<i>Picea excelsa</i>)	1.21	"
Beech (<i>Fagus silvaticus</i>)	1.58	"
Birch (<i>Betula verrucosa</i>)	2.29	"
Poplar (<i>Populus tremula</i>)	1.39	"
Ash (<i>Fraxinus spec.</i>)	1.30	"
Willow (<i>Salix spec.</i>)	1.17	"
Alder (<i>Alnus glutinosa</i>)	1.89	"
Maple (<i>Acer campestre</i>)	1.62	Beckmann (1915)
Elm (<i>Ulmus campestris</i>)	2.04	"

(2) THE FATE OF THE WOOD COMPONENTS IN XYLOPHAGOUS INSECTS.

It is quite clear from this survey of the analyses of wood that, apart from the ligno-cellulose complex, there are considerable quantities of less complex substances, such as the hemicelluloses, starch, sugars and proteins, which could all be utilised as food. As to the ligno-cellulose complex, recent research on its fate in the gut of insects has shown definitely that the lignin part passes through the gut quite unaltered, while the cellulose in certain insects undergoes splitting through the activity of enzymes.

In dealing with the literature on the enzymes of xylophagous insects which can break down the different components of wood (other than proteins), it is found advisable to consider firstly the cellulose-splitting enzymes, secondly the hemicellulose-splitting enzymes, thirdly the enzymes attacking the simpler carbohydrates present in the wood and lastly the proteolytic enzymes.

(a) Cellulose-splitting enzymes.

Cellulose is one of the substances which has long been known to be very resistant to the action of digestive juices. In vertebrates the breakdown of cellulose is not the work of a cytase, but it is mainly brought about by bacteria present in the different parts of the intestinal tract (Woodman, 1930).

In 1899 it was shown by Biedermann and Moritz that the digestive juice present in the crop of the snail, *Helix pomatia*, is able to digest cellulose. Since then a number of other instances of cellulose digestion in invertebrates have become known, e.g. in the ship-worm, *Teredo* (Harrington, 1921; Potts, 1923; Dore and Miller, 1923; Lazier, 1924; Miller and Boynton, 1926), in *Bankia* (Boynton and Miller, 1927) and in *Pterocera* (Yonge, 1932).

Experiments designed to demonstrate the presence of cellulase in insects always gave negative results up to 1919. Jordan (1913), after a thorough study of the literature on this subject, answers the question: "Is a cellulose-dissolving ferment (cellulase or cytase) present in insects?" in the negative.

Between 1919 and 1930, however, instances of cellulose digestion have been reported by Biedermann (1919) in different species of the locusts of Acridiidae (e.g. *Gomphocerus*, *Stenobothrus*); by Bělehrádek (1922) in the stick insect, *Dixippus morosus*; by Hering (1926) in the larvae of the lepidopteran *Cemiostoma* and by Smith (1926) in various members of the hemipteran families Capsidae and Coccidae.

Notwithstanding these clear cases of the presence of cellulase in insects, many reviewers of the subject, such as Oppenheimer (1925), Waksman in Pringsheim (1932), and Yonge (1926), still maintain the view that digestion of cellulose by the digestive juices of insects is absent, particularly where the cellulose is in the form of ligno-cellulose. It is probably owing to this controversy among physiologists concerning the fate of cellulose in insects that the theory of the digestion of this substance with the help of micro-organisms has gained ground.

Undoubtedly the interpretation given by Campbell (1929) of his results on the anobiid *Xestobium* beetle was inspired by this hypothesis. Campbell demonstrated

the disappearance of cellulose during the passage of wood through the alimentary canal of this insect. By comparative analyses of a sample of the heartwood of the oak on which *Xestobium* larvae occur and of the frass, he found a decrease in cellulose content from 53.2 to 39.0 per cent. From this the conclusion was drawn that micro-organisms play a vital part in the digestion of wood.

Since 1930, the evidence on the digestion of wood by the secretions of insects has increased. Falck (1930) carried out experiments on *Hylotrupes bajulus* (fam. Cerambycidae). During its larval life, which lasts up to 9 years, this species lives in pinewood. According to Falck, the excreta are always free from bacteria; nor are there any micro-organisms in the gut or in the borings. By comparing the analyses of the unattacked parts of the wood and of the excreta, Falck showed that the cellulose content of the latter is 12.3 per cent. lower than that of the sound wood (the ash and lignin content being considered constant). Owing to the fact that the partial breakdown of the wood by these larvae is not accompanied by any coloration (browning) of the excreta, Falck supposes that no destruction of the lignin cellulose complex takes place. Falck designates this type of attack on wood as "Simulatdestruktion," as opposed to (1) real destruction caused by fungi (Falck and Haag, 1927; Campbell, 1932), where the cellulose disappears totally, leaving the lignin intact, and to (2) corrosion, where the lignin first disappears, and the cellulose is only attacked very late (Falck and Haag, 1927; Campbell, 1932).

Ripper (1930) demonstrated cellulose digestion in insects by comparing analyses of food and excreta of some wood-eating larvae and by carrying out enzyme tests on the digestive juices of insects.

For the beetle *Xestobium rufovillosum* (Anobiidae), Ripper confirms Campbell's results on the disappearance of cellulose. In different species of cerambycids, viz. *Cerambyx cerdo*, *Leptura* spec. and *Rhagium bifasciatum*, the presence of a cellulose-splitting enzyme has been detected in qualitative tests by microscopical examination of sections of lettuce ribs after the action of digestive juice. Quantitatively the cellulase has been measured by weighing the cellulose before and after the juice acted on it.

The first-named cerambycid species is entirely free from micro-organisms. Ripper therefore infers that the presence of a cellulase in these three cases, and in the *Xestobium* species examined by him and by Campbell, is quite independent of the occurrence of micro-organisms. Mansour and Mansour-Bek (1933, 1934) showed the presence of a cellulase in the digestive juice of a cerambycid larva, *Macrotoma palmata*, qualitatively by dissolution of the cell walls of date stones and lettuce ribs. In quantitative experiments, the strength of the cellulase action was determined by titrating the formed sugars. The strength of the cellulase of this species was found by these authors to be the same as that of the snail, *Helix pomatia*, studied extensively by Karrer (1925).

It is evident from this survey that some insect larvae are capable of digesting cellulose by means of their own enzymes. The action of the digestive juices on pure cellulose (Ripper, 1930; Mansour and Mansour-Bek, 1933, 1934) shows the presence of a cellulase, whereas the experiments of Campbell, Falck and Ripper show that, with the help of this cellulase, the insects can attack the cellulose from wood. Falck

believes that the larvae of *Hylotrupes* only attack the cellulose not bound to lignin. Ripper, on the other hand, from the darker colour of the excreta of *Xestobium* concludes that lignin is set free during the passage of wood through the gut; he also believes this to be the case in the *Xestobium* species examined by Campbell, where the frass is described as being dark in colour.

Falck (1930) mentions that the larvae of *Hylotrupes bajulus* grind their food into a very fine powder. Mansour and Mansour-Bek (1934), in their study of *Macrotoma palmata* and *Xystrocera globosa*, record the occurrence of a well-developed proventriculus in the former species, and suggest that perhaps in the larvae of *Macrotoma* the food material must be in a very finely divided condition for the proper action of the cellulase. Microscopical examination of the sizes of the food particles in the alimentary canal of the two species gives support to this suggestion.

(b) Hemicellulose-splitting enzymes.

Only a few data are available on the digestion of this group of substances. As early as 1905, Seillière, struck by the fact that some beetle larvae are able to live in wood, compared the pentosan content in food and excreta of the larvae of a cerambycid, *Phymatodes variabilis*, which bores its passage between the bark and the wood of the beech. Whereas the food is likely to have an average content of 21.22 per cent. pentosans (bark 18.9 per cent., wood 23.54 per cent.), the excreta contain only 18.48 per cent. From these figures Seillière concludes that pentosans are utilised as food. He showed also that the watery extract of the gut and its contents has a digestive action on the pure pentosan of poplar. The products of digestion reduce Fehling's reagent and also give the pentose reaction with phloroglucine and with orcin. Moreover, he was able to obtain an osazone, which was probably xylosazone, though its melting point was not quite identical with that of pure xylosazone. Seillière attributes this difference to impurities in the digestive mixture.

This fundamental research of Seillière, though often quoted, has not received the attention it deserves, and it was not until 1929 that a similar difference in pentosan content between food and excreta was demonstrated by Campbell, who, working on *Xestobium* spp., found a decrease of from 21.7 to 16.5 per cent. Falck (1930) in his work on *Hylotrupes bajulus* finds also a small decrease in the hemicelluloses. In this case the hexosans diminished from 15.30 to 12.64 per cent., while the quantity of pentosans was the same in food and excreta. Ripper (1930), from his work on *Cossus cossus*, also suspects the digestion of xylan by this species.

In this connection reference must be made to the work on the digestion of lichenin or reserve cellulose which is considered by some authors (Oppenheimer, 1925) as belonging to the hemicelluloses and by others (Schorger, 1926) as closely related to the starch group. Jewell and Lewis (1918) record the general occurrence of lichenase in all invertebrates including insects, and Ullmann (1932) has actually succeeded in demonstrating the presence of this enzyme in *Cossus cossus* where Ripper (1930) found no true cellulase.

(c) *Enzymes splitting starch, sugars and proteins.*

Starch. In the beginning of this section attention was drawn to the high starch content of some kinds of wood, as compared with others which are almost starch-free (see Table II). As early as 1903 Mer made some remarkable observations on the relation existing between the starch content of wood and the liability to attack by species of *Anobium* and *Lyctus*. This author succeeded in de-starching the wood by different methods, and found that such wood becomes quite immune from the attack of these insects. He concluded that starch is really the food which these species derive from wood.

Mansour and Mansour-Bek (1933), working on the digestive juice of the *Xystrocera globosa*, failed to find any trace of cellulase. Moreover, modified cellulose (treated with zinc chloride or Schweizer's reagent), which is generally more readily digested by the suitable enzymes than natural cellulose (Karrer, 1925), is not attacked at all. A strong amylase is present in the digestive juice. The larvae of *Xystrocera* appear to derive their food from carbohydrates other than cellulose. Analyses were accordingly made of the sapwood of *Albizzia lebbek*, on which *Xystrocera* occurs. It was found that this wood contains 3.9 per cent. starch (apart from the other sugars), an amount which is considered adequate as a carbohydrate supply for the larvae of *Xystrocera*. The heartwood of *Albizzia*, on the other hand, contains only 0.5 per cent. starch. This would explain the fact that *Xystrocera* larvae never penetrate into the heartwood.

Analyses of the wood of *Poinciana* and *Tamarix* also showed a high starch content (4.5 and 4.4 per cent. respectively). These two kinds of wood are attacked by the larvae and adults of *Sinoxylon ceratoniae* and *Bostrychoplites zickeli*, which also occur in the smaller branches of *Albizzia lebbek*. The beetles in question thus appear to be similar in their feeding habits to *Xystrocera globosa*.

Wilson (1933) showed the relation between the starch content of timber and its liability to attack by *Lyctus*, the powder post beetle. He studied qualitatively, by means of the iodine test, the starch content of timber which had been subjected to various modes of seasoning. Apart from the dependence of the amount of starch on the species of tree and on the time of felling, he found that when seasoning takes place by desiccation or excessive heat so that the cells die quickly, the amount of starch remains unaltered, whereas after delayed drying under moderate conditions of temperature, or after immersion in water, the starch content diminishes, or disappears entirely. The delay in drying enables cell activity to continue, and, as a result, the starch is used up. This view was supported by measurements of the respiratory exchange of appropriate pieces of wood.

Wilson was struck by the fact that the attack of *Lyctus* on certain hard woods, and only in the sapwood, was definitely localised. "Microscopic examination by means of thin sections of both the infested and the uninfested sapwood (of ash) revealed the fact that starch was present in abundance in the 'worm-eaten' wood, but that it was absent from the rest of the wood" (*loc. cit.* p. 677). This relation of the localisation of the larvae to the starch-containing wood was confirmed for other

pieces of infected wood of different kinds. Microscopic examination revealed the total absence of starch in the wood particles present in the rectum and in the greater part of the faeces found in the tunnels in the wood. Wilson concludes that starch is used as food by the *Lyctus* larvae. The liability of wood to attack by *Lyctus* is determined by its starch content, and samples of wood in which total starch depletion has taken place are immune to *Lyctus* attack, whereas the rapidly air-dried and steamed samples, in which starch has been retained, are attacked. The different kinds of wood on which *Lyctus* occurs (e.g. ash, oak, sweet chestnut, walnut and willow) all show a great amount of starch in their sapwood, which alone is attacked.

Although Wilson's work was all done by means of the iodine test for starch, and no quantitative determinations were made, his work certainly demonstrated the existence of a definite relation between the chemical composition of wood and the liability to attack by *Lyctus*.

In the light of this research Wilson points out that the results obtained by Campbell on analyses of food and excreta of *Lyctus*, in which Campbell failed to find any difference in composition, may be due to the fact that starch was not determined, and so the decrease in quantity escaped notice. Campbell's results may, however, be due to the fact that the "frass" which this author takes as excreta had not all, or even a great portion of it, passed through the alimentary tract of the insect. From our observations on similar wood-eating insects (*Sinoxylon* and *Bostrychoplites*) we can definitely say that a great proportion of the "frass" of such insects consists of wood particles which show no signs of having been passed through the alimentary canal.

Concerning the mechanism of the digestion of starch, Ullmann (1932) concludes from her experiments on different species of insects, including *Cossus cossus* and *Rhagium* spp., that starch grains can only be digested if previously broken up by the mouth-parts. This would mean that a great portion of the starchy material taken in by *Lyctus*, etc., is not utilised as food. On the other hand, Bělehrádek (1922) states that whole starch grains are attacked by the saliva of *Dixippus morosus*.

Soluble sugars. The information available on the quantity of these substances in wood is still more scanty than that concerning starch, but nevertheless in a few cases the data indicate that this fraction of the wood components is of importance.

Ripper (1930), working on the question of cellulose digestion by xylophagous insects, has shown that no cellulose is broken down by the secreted juices of *Cossus cossus*. On the other hand, the comparison of analyses of food and excreta showed a marked decrease in the alcohol-benzol extract, and a decrease in reducing sugars from 2.27 per cent. in poplar wood to 0.0 per cent. in excreta of *Cossus cossus*. Ripper is accordingly convinced that these larvae derive their necessary carbohydrates, at least partly, from the soluble sugars. This conclusion is supported by his remarkable observation that *Cossus cossus* larvae when fed on materials rich in sugars, such as beetroot, complete their development in a year, whereas in their natural habitat the imaginal stage is reached after 2½ years.

Mansour and Mansour-Bek also found that the content in soluble sugars of some kinds of wood, like the starch content, is quite considerable (Table II). They

demonstrated the presence of saccharase and maltase in the gastric juice of *Xystrocera globosa* which lives on such kinds of wood (*Albizia lebbek*), and conclude that this insect, as well as some bostrychid beetles (*Sinoxylon ceratoniae* and *Bostrychoplites zickeli*) which live on similar species of wood, depend on the starch and soluble sugar content for their carbohydrate supply.

As early as 1920 Boodle and Dallimore pointed out that *Dinoderus minutus*, a bostrychid beetle which attacks bamboo, is very likely seeking sugar. These authors, trying to explain the fact that bamboos well soaked in water are usually untouched by boring beetles, carried out qualitative experiments on the feeding habits of this insect and concluded that lack of sugar is very likely the cause of the immunity. The occurrence of this insect in food substances rich in sugar, for example yams, ginger roots and grain, may be quoted in support of the conclusions of these authors, but quantitative experiments might show that starch is also an important food source.

Proteins. To our knowledge there is no evidence for the presence of proteolytic enzymes in any of the wood-eating insects. Uvarov (1929, p. 266), in his discussion of the nutrition of wood-eating insects, mentions that "the most difficult problem is not the digestion of cellulose, but how to discover the means by which the deficient nitrogen is obtained."

The relatively high content of different kinds of wood in nitrogenous substances (Table III) and the general occurrence of proteolytic enzymes in the insect species which have been investigated (Uvarov, 1929) make it probable that wood-eating insects derive their nitrogenous substances directly from the wood. A similar conclusion has been arrived at by Ripper (1930).

(d) Discussion.

The experimental work has demonstrated the ability of wood-eating insects in general to digest various components of wood with the help of their own enzymes. Reference has been made earlier (p. 368) to the case of Protozoa-harboured termites and lamellicorn larvae where actually wood particles are taken in but intestinal micro-organisms are also utilised as food source. At present we have no experimental data as to what such insects subsist on apart from the micro-organisms they digest (Wiedemann, 1930). Uvarov (1929, p. 265) has concluded from the feeding experiments of Cleveland (1928) that termites "may be able to extract some nutritive substance from cellulose without the aid of symbionts." We are of the opinion that the enzymes of the Protozoa-harboured termites may differ in different species. In this way the findings of Cleveland could be explained. Termites with a cellulase, for example, could live indefinitely on wood which is poor in the less complex carbohydrates, even after the removal of their intestinal Protozoa, while those without cellulase could only live on the soluble sugars and starch in the wood they attack and supplement their diet directly from the culture of Protozoa which they harbour. This is supported by the discovery of Holdaway (1933) that the most serious termite in Australia is free from Protozoa.

The Protozoa-harbouring termites and the lamellicorn larvae which utilise their intestinal micro-organisms as a direct food supply are analogous to the ambrosia beetles and the Ciricidae (Buchner, 1930), to insects living on rotting wood such as *Sciara* (Baumberger, 1919), and to some Tipulidae (Buchner, 1930) which live mainly on the fungus growing in their burrows or on micro-organisms in the material they ingest.

The duration of the larval stage of some wood-eating insects, for example, some members of the Cerambycidae, has often been referred to by biologists as extremely irregular. In Munro's words (1928, p. 7): "it may last from a few weeks or months to one or two years under normal conditions but in some circumstances it may be prolonged to an extraordinary degree and extend to thirty or forty years." It appears to Munro that the dry nature of timber is the chief factor in causing this slow development. Wilson's work has demonstrated that the prevalence of starch in certain kinds of commercial timber depends on the method of seasoning. It is also believed that the content varies according to the time of felling. In view of the experiments of Ripper (1930) on *Cossus cossus*, we suggest that the concentration of food material in the wood on which such larvae live may also be a very important factor in determining the rate of growth.

That chemical composition is one of the most fundamental factors which determine the susceptibility of any kind of wood to the attacks of insects, nobody now can question. This certainly explains why in many cases sapwood is rendered useless while the heartwood of the same block is quite untouched. A better knowledge of the enzyme secretions of the important wood pests coupled with an accurate analysis of the kinds of wood of commercial value must lead to means of control. Wilson (1933) has already gone ahead in this direction. Munro (1928, p. 9) writes that: "It is important to observe that with the possible exception of *Hylotrupes*, all the longicorn beetles commonly imported (into England) prefer unseasoned timber as a breeding ground and for that reason they rarely increase in numbers in timber yards and the injuries they cause do not extend to other timber in the vicinity. Where timber yards and saw mills are situated in a forest district, longicorn beetles introduced in imported timber may become established in the district." Among the cerambycids, we know now of the regular occurrence of cellulase and we know through the work of Falck (1930) that *Hylotrupes bajulus*, the species to which Munro refers, is able to secrete cellulase. This probably explains its ability to live and develop in seasoned wood. Other species, devoid of such an enzyme, can probably only spread in unseasoned wood or "timber in the round."

V. SUMMARY.

1. The relation between certain insects and the intracellular micro-organisms they harbour is obscure and cannot be described as symbiotic for the following reasons:

(a) Sterile mycetomes are known to occur in some species closely related to others with heavily infected mycetomes.

(b) Defloration experiments have been carried out successfully without any harmful effect to the host insect.

2. Intracellular micro-organisms in xylophagous insects cannot be considered as playing an important role in the digestion of wood for the following reasons:

(a) In weevils with wood-eating habits which have intracellular micro-organisms, the latter only pass into the lumen of the gut of their host during the adult stage when the insect is not feeding on wood.

(b) Some wood-eating species of insects harbour intracellular micro-organisms, while closely related species with similar feeding habits are free from these.

(c) The intracellular micro-organisms of some wood-eating forms have been cultivated *in vitro* and found to be unable to break down cellulose.

3. The relatively high content in nitrogenous substances of different kinds of wood, and the general occurrence of proteolytic enzymes in insects, make the assumption that intracellular micro-organisms fix atmospheric nitrogen for the use of their host superfluous.

4. The extracellular intestinal micro-organisms of certain lamellicorn larvae and of some termites play no role in breaking down cellulose for the use of their host. They are utilised as a direct food source. Such insects are therefore better referred to as micro-organism-feeders.

5. True wood-eating insects derive the necessary carbohydrates from the wood they live on through the activity of their own enzymes. The enzyme complex in such insects has been found to vary from species to species.

6. The carbohydrate components of wood vary in quantity in different kinds of wood.

7. Some true wood-eating forms depend upon starch and soluble sugars for their source of carbohydrates. Such insects have no cellulase and can consequently only live in kinds of wood comparatively rich in starch and sugars.

8. Other true wood-eating forms secrete cellulase and are therefore able to live in kinds of wood comparatively poor in starch and soluble sugars.

9. The occurrence of hemicellulase has been demonstrated only in a very few cases and the value of the hemicelluloses as food for wood-eating insects has not been thoroughly investigated.

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THE DERIVATION OF THE NITROGEN OF CROP PLANTS, WITH SPECIAL REFERENCE TO ASSOCIATED GROWTH

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CONTENTS.

	PAGE
I. Decline and desuetude of unitary theories of plant nutrition	383
(1) Historical and general	383
(2) The development of modern views on the nitrogenous nutrition of plants	384
II. Evidence for the utilisation by non-leguminous plants, of substances originating from the activity of nodule-bearing plants	388
(1) Pot experiments	388
(a) Uptake of nitrogen	388
(b) Uptake of phosphorus	395
(2) Accessory factors and the growth of plants; a suggested rôle for leguminous plants	395
(3) Associated growth in practice	397
(a) "Competition" between legumes and non-legumes	397
(b) Cover crops. The value of leguminous crops in a rotation	401
III. Tables summarising experiments on associated growth	403
IV. Conclusion	405
V. Summary	406
References	407

I. DECLINE AND DESUETUDE OF UNITARY THEORIES OF PLANT NUTRITION.

(1) HISTORICAL AND GENERAL.

EARLY investigators searched for an active "principle" in the soil to account for plant growth. Glauber (1656) concluded that this principle was saltpetre (cf. Nicol, 1932)—a view supported by experiments by Mayow (1674), whose findings bear a curious resemblance to the conclusions of some modern work on the course of the seasonal production of nitrates in field soils. Külbel (1741), however, looked to a *magma unguinosum* obtainable from humus as the source of fertility. Wallerius (1761) concluded that humus, being *homogeneous*, is the source of the food of plants,

their *nutritiva*, while the other soil constituents are *instrumentalia*, dissolving and attenuating the food mixture proper so as to fit it for being taken up by the plant.

Nevertheless, a defect of many early theories was that they were purely monistic: they postulated some exclusive principle. It was not until the phlogistic period that a plurality of sources of soil fertility was recognised. According to modern views, the soil, potentially a reservoir of plant food, is at any given moment functioning chiefly in relation to the plant as a support, as a substrate for the reception of added nutrients such as manures, and as an ingenious mechanism for the transport and interchange of existing nutrients carried by the soil atmosphere and by the soil water supply. (For a review of the theories of plant nutrition see Russell, 1932.) These nutrients, though centred around the substances symbolically represented by that magic trinity of letters N-P-K, now seem likely, as regards the number of elements involved, to be limited only by the range of atomic numbers. Yet, forms in which the chief known elements are considered to be available to plants are limited in number. For the majority of compounds it could be said that the simpler they are and the more familiar they were to students in the elementary stages of inorganic chemistry, the more likely have they been held to be highly available to plants. Thus, nitrogen would be taken up by plants as nitrate, more rarely as ammonia; phosphorus as phosphate; potassium as chloride or sulphate. Indeed, some of the latest developments in the manufacture of fertilisers have resulted in mere permutations of these radicles to give an ammonium phosphate intermixed with some equally simple potassium salt.

There are exceptions to the simplicity: in the case of basic slag it seems that neither can its calcium be regarded simply as "lime" nor are the phosphorus compounds available as simple phosphates. Some of the complex chemistry of the compound phosphates may have to be invoked before an understanding can be reached of the utility to plants of the phosphorus compounds in basic slag. Study of the uptake of potassium and sodium compounds seems as yet uncomplicated; problems of rock weathering and soil decomposition, at least as far as the alkali-metal nutrition of plants is concerned, appear to reduce themselves ultimately to the simplest ionic terms. Can we say the same for the nitrogenous nutrition of plants?

(2) THE DEVELOPMENT OF MODERN VIEWS ON THE NITROGENOUS NUTRITION OF PLANTS.

Simple water-soluble compounds of nitrogen are few; they consist of the series of oxidised acids and anhydrides belonging to the nitrates, nitrites, and hyponitrites; hydrides such as ammonia, hydrazine, hydrazoic acid; and intermediately oxidised bodies such as hydroxylamine and urea. The study of more complicated bodies than these would take us into deep waters of organic chemistry. It has been a very general assumption that all these substances—including simple as well as complex organic compounds of nitrogen—that are not actually toxic to plants, would if they occurred under natural conditions of field and forest be broken down, and lastly oxidised, to the form of nitrate. According to this view, nitrate is the "elemental"

form of food nitrogen for the ordinary plant: that is, the only simple form which it would encounter and the only harmless compound of nitrogen which it would take up. Some reservations were always made; thus, leguminous and certain other plants can utilise combined nitrogen resulting as a product of the activity of the nitrogen-fixing bacteria associated with the nodules, but few writers admitted the possibility of non-leguminous plants taking up compounds of nitrogen that had not undergone a change into nitrate.

Is this unitary creed too simple? At one time Liebig taught that the nitrogenous requirements of all plants were satisfied by ammonia (as such). In the light of our present knowledge there is no doubt that Liebig's "ammonia" was no more than an intelligent guess. A belief in the superior value of ammonia to plants was held for some time in France, as a result of observation by the early agricultural chemists. Boussingault was so much impressed with the effects of guano and of rotting manure that he regarded ammonia as the essential form of nitrogen. "Kuhlmann (1847) even went so far as to suggest that nitrate needed to be reduced by rotting organic matter before its nitrogen became available to the plant" (Crowther, 1934).

Liebig was wrong in being too sweeping, but it is reasonable to ask whether some of our present-day teachings concerning the nutrition of plants do not err through excessive simplification. Justification for the view that nitrate is in nature the chief, if not the only, nitrogenous building material of plants has been derived from a consideration of the nitrogen content of agricultural soils, in tropical as well as temperate climates. In such soils the content of nitrogen as nitrate, though very variable, may rise to 40 or more parts per million of soil. These amounts seem small, but it should be remembered that only about 15 per cent. of the soil is water, so that the nitrate nitrogen in the soil water, considered as a nutrient solution, is present in concentrations up to about 300 parts per million of water. To represent a concentration of calcium, sodium, or potassium nitrate these figures must again be multiplied by six or seven. Ammonia, on the other hand, is usually present in much smaller amounts in arable soils.

Until quite recently work on the soil's content of "mineral" (inorganic) nitrogen had been carried out on arable soil, frequently on fallow soil, and in any case sampling was performed in interspaces—the immediate neighbourhood of mature plant roots has been avoided. Hence it will be appreciated that the soil's content of nitrate was evaluated mainly in the absence of the plant. Reuszer (1931) found that the nitrate content of a grassland soil was minute and nearly constant. After this same soil had been ploughed up, its nitrate content increased greatly, showing, however, seasonal fluctuations. If plant roots take up ammonia, the fact would hardly have been detected by the usual methods of sampling arable land; in places bare of roots ammonia would have every opportunity of undergoing microbiological conversion to nitrate. Even a much fuller knowledge than we now possess of the soil nitrate "gradient" in the neighbourhood of plant roots would not assist us to determine whether the formation of nitrate is primarily of value to plants, or whether it represents a stage reached by nitrogenous compounds in the absence of living roots.

Other nitrogen compounds, such as proteins and their decomposition products, have been assumed to require breaking down by simplifying processes such as ammonification and nitrification; and literature of these processes forms an impressive part of the literature of soil biology and soil chemistry. Soil chemistry had its origin in Germany, France, and England, and spread to include investigations elsewhere in the temperate zone and in the tropics. It was therefore natural that findings relating to agricultural soils of these well-investigated regions should be taken as typical of most types of land, and that plants should be assumed to nourish themselves upon the nitrate which was thus recorded to be widespread and predominant in the nitrogen make-up of soils. In Sweden, however, Hesselman (1925-6) showed that in certain types of forest soils, composed largely of humus layers arising from dead leaves, the principal nitrogenous end-product of the soil biochemical processes was ammonia; further, the vegetation peculiar to these soils was well able to utilise this ammonia for growth without nitrification being necessary. In fact, some of these forest soils were almost incapable of turning ammonia into nitrate.

Hesselman is a worker who adopts a method of research that may for convenience be called the "naturalistic," or better, the "realistic." As far as possible he studies the soil just as it is, without adding anything to it or using it to grow plants under greenhouse or other artificial conditions. The natural ecology of the soil is for him the best plant indicator. Very different principles are followed by the workers who add and subtract substances to soil or to culture solutions in an attempt to elicit the response of a plant, the choice of which is often arbitrary, based upon the convenience of the worker, or upon some supposed typicality in the plant. This second method of attack has been very extensively employed; in this connection it will suffice to mention the work of Prianishnikov. In a series of papers, some of them difficultly accessible outside Russia, Prianishnikov has claimed that ammonia is efficiently utilised by certain common agricultural plants (*e.g.* peas and beetroot). Prianishnikov (1933) has written the first part of a summary of his own work. For discussion of the physiological aspects of vegetable nutrition see Crowther (1934), Onslow (1931), and Robinson (1929). Evidence is conflicting as to whether ammonia or nitrate is the more efficiently utilised by plants, but there is considerable agreement that they can use either source.

The overwhelming importance that has been attributed to nitrate would seem to have arisen largely from the unequal extent to which arable land, grassland, and forest soils have been investigated. If we admit that ammonia, as well as amino- and amido-nitrogen, is important, not merely in the nutrition of the flora of particular areas of forest but also throughout the wide expanses of grassland and prairie, the nitrate castle crumbles and we are no longer able to defend the position that there is a unique source of nitrogen for non-leguminous crops.

Grassland offers us an opportunity for studying the uptake of nitrogen under conditions such that all the available soil is penetrated with plant roots. Hall, Miller, and Gimingham (1908) were among the first to break away from a pre-occupation with nitrate as the principal source of nitrogen for plants. They

suggested that grass in acid soils "must, in the main, utilise the ammonium salts without previous change." Surprisingly little work has been done since 1908 on the mineral nitrogen relationships of grassland. Richardson's (1933) work on the fate of additions of ammonium salts to Rothamsted grass soils of various reactions has confirmed this supposition that grassland vegetation can directly assimilate ammonia.

The weight of observational evidence in the ordinary arable field has been supposed to support the view that nitrogenous manures must be supplied in the form of nitrate or must be converted to nitrate before being available to agricultural crops (cf. Beaumont and Moore (1933) for a summary). It is common knowledge that if undecomposed or partly decomposed nitrogenous manure, not containing much nitrate, is added to soil just before planting a crop, the crop usually suffers therefrom. A counsel of perfection is to apply farmyard manure always to the previous crop—jam yesterday but never jam to-day. Farmyard manures and the like contain comparatively large amounts of the simpler organic compounds of nitrogen, derived from decomposition of protein. Some at least of these simpler substances should be economically available to plants, if Prianishnikov, Mevius, Mothes, and others are right. Yet age-long practice counsels against the application of fresh dung. The mere absence of nitrates might account for a starved plant, but could not harm the plant to the extent commonly anticipated.

Two explanations are available for the harmful effect of fresh nitrogenous manure. One takes account of the carbohydrate material—usually straw or other plant residues—which is added with most of the common farmyard manures or simply as "green manure." According to this theory, when carbohydrate material (with the nitrogen compounds contained in the plant residues as well as that derived from urine and faeces) is decomposed, swarms of fungi and bacteria pullulate upon the carbohydrates. Kalatshikov (1928–30) has shown that in three Russian soils, in presence of pure starch, the nitrogen of ammonium sulphate and of calcium nitrate can be largely taken up in the tissues of micro-organisms (cf. Murray, 1921). They "lock up" not only nitrogen from the manure but also the soil's own nitrogen which is thus rendered unavailable to plants, until such time as the micro-organisms themselves decompose (Jensen, 1932; Doryland, 1916). This explanation is undoubtedly well founded as far as it relates to strawy dung and new composts, and to the repressive effect of ploughing-in undecomposed carbohydrate materials such as fresh straw. But it fails to account for the behaviour of amendments not notably rich in carbohydrates, such as human night soil and poultry manure, about which the expression "hot" is often used. Some other explanation must be sought, and hitherto the absence of sufficient amounts of nitrate has been taken—somewhat inadequately—to account for the nutritive aspect of the question.

Barritt (1933*a*) has put forward a theory based on numerous experiments, according to which the repressive effect of fresh organic nitrogenous material on the growth of nitrifying bacteria is due to the accumulation of carbon dioxide and ammonia, resulting from the activity of micro-organisms. The soil atmosphere is

vitiated and the partial pressure of oxygen is reduced. According to Barritt, an unfavourable result from this unnatural atmosphere is a suppression of the formation of nitrates by the nitrifying bacteria. Winogradsky claimed that nitrifying bacteria could not function in presence of organic matter. Barritt takes a much less extreme view of the restrictions of their biotic requirements. The force of Winogradsky's claim has been considerably reduced since the discovery of ammonia-oxidising bacteria capable of living on organic media (Cutler and Mukerji, 1931; Cutler and Crump, 1933); indeed, it would seem absurd to credit the nitrifying bacteria with any activity in soil if they are as exacting as Winogradsky claimed. Barritt's view accords well with what one would expect to find in soil: he has successfully resolved a number of apparent contradictions respecting the natural course of nitrification.

Barritt's theory thus brings us back to the question of the value of ultimate nitrification. He thinks that nitrification is desirable, but he did not consider, in that paper (1933*a*), the question of how conditions will affect the *plant* when so much carbon dioxide and ammonia has accumulated as to stop nitrification. It seems highly probable, nevertheless, that a poorly oxygenated soil atmosphere and soil solution, rich in carbon dioxide and free ammonia, would be unfavourable to plant growth on account of the effects upon root respiration. Willis and Rankin (1930) have demonstrated injury to plant roots arising from liberation of free ammonia when cotton-seed cake was used as fertiliser. In agreement with Barritt, it was found that such excessive ammonia could not be nitrified. Jashnova (1930), however, found that nitrification of high amounts of ammonia was delayed only if phosphates were present in insufficient quantity. It is understandable that a partly asphyxiated plant would be unable to profit from any nitrogenous constituent of its environment. On this view, the plant cannot profit from organic manures while their organic matter is in a state of rapid decomposition. Thus, a high degree of nitrification may imply little more than that those decomposition processes which pollute the soil atmosphere have reached a term. The plant may, however, use nitrate as a source of oxygen (Barritt, 1933*b*). The formation of nitrate from rapidly decomposable organic nitrogenous manures would, on this view, be an index of substantial cessation of the processes of decomposition. It would not necessarily be an end desirable in itself.

II. EVIDENCE FOR THE UTILISATION BY NON-LEGUMINOUS PLANTS, OF SUBSTANCES ORIGINATING FROM THE ACTIVITY OF NODULE-BEARING PLANTS.

(1) POT EXPERIMENTS.

(a) *Uptake of nitrogen.*

The manurial use of complex protein material such as dung, horn, feathers, carrion, and so on is of high antiquity, but owing to the preoccupation of soil chemists with the "nitrate theory," very little exact work has been done on the

parts taken by organic nitrogen compounds. Latterly, simple nitrogen compounds of carbon—urea, cyanamide—have assumed commercial importance, but their fertilising function in soil has been assumed to be related to the effectiveness and rate of their ammonification, with subsequent nitrification. In the complex biodynamical equilibrium of soil, it may be true to say that urea and cyanamide are ultimately nitrified, as are all other nitrogen compounds, when not taken up, at some stage or other of their decomposition, by the plant. But just because the soil is so complex and biologically active it is difficult, if not impossible, to determine exactly what happens within it to a decomposable addition.

Recourse must therefore be had to the simpler conditions offered by sand or liquid cultures in small vessels. A review of most of the early work with single plant species was made by Hutchinson and Miller (1911). In their original investigations these authors showed that plants (peas and wheat, separately grown) can absorb some water-soluble organic nitrogenous compounds.

Suggestive results have been obtained from experiments on the growth of two or more species simultaneously in one culture flask or pot. Especially valuable have been the experiments on the associated growth of leguminous with non-leguminous plants. Agriculturalists rarely aim at growing two or more non-leguminous species together, unless one or more species of legumes be present. The culture of "Maslin," "mashlum"—mixed cereals—is an exception, as is the growing of a weedy crop. Sometimes two or more grasses are grown in mixture, but much more frequently one or more clovers or other legumes are incorporated in a mixture of grasses. Forage crops, which, like the yield of grassland, are intended solely for consumption by animals on the farm, and not for sale at market, also commonly comprise several species. When they do, at least one legume is an almost invariable component. (Intercropping in market gardening on highly manured land is a distinct kind of operation which need not be considered here.) It is, moreover, a common practice to under-sow legumes in a cover crop of cereals (see section "Cover Crops," p. 401).

Thus practices have developed which suggest that leguminous plants have a singular rôle in agricultural economy. The Roman writer, Varro, remarked upon that property of leguminous crops which we should nowadays call restorative: that is, he noted that a legume crop increased the fertility of soil and benefited a succeeding non-legume. This valuable property of legumes was systematically utilised in the process of rotation of crops, whereby once in a period of years—usually every fourth year—clover or beans were sown. It was not until 1886 that the outstanding peculiarity of leguminous plants was discovered to be due to their ability to capture atmospheric nitrogen by the aid of bacteria living within the root-nodules. Somehow, by a process not yet understood, the joint action of nodule bacteria and plant tissue made available to the leguminous plant that atmospheric nitrogen which was unavailable to all high animals and to nearly all other higher plants. Even in a poor soil, the legume thus became rich in nitrogen—richer than most non-legumes—and, dying, left a nitrogen-rich root residue which decomposed and acted as a source of available nutrients to its successors.

This property of legume roots of increasing the soil's fertility by their subsequent decomposition has no obvious bearing upon the question of associated growth in conditions where the beneficial effects of the legume often occur before much root decomposition can have occurred. Only during the present century have conscious attempts been made to relate the two things.

It is not overstating a case to say that a mixed vegetation is symbiotic. Leather (1897) seems to have been the first to appreciate the possibility of this being so. He wrote: "The question naturally arises, are the Papilionaceæ able to assist in any way the plant of another natural order *which is growing alongside them.*" Observations of the Indian practice of growing gram (*Cicer arietinum*) and wheat together, led to this speculation. Farmers have often remarked on enhanced vigour in non-legumes growing with legumes, and it would appear that science once again has lagged behind practice. It was from consideration of field growth of mixtures of Canada peas and oats that J. G. Lipman was led to make in 1908 what appear to be the first pot experiments to have a bearing upon the question of transfer of nutrients in associated growth. Lipman claimed (1912) that his interest in the subject arose prior to 1905, but the first (albeit an anonymous) publication that can be ascribed to him appeared in 1910*a* and related to experiments made in 1909. It was not until 1912 that he published the results of the experiments made in 1908, when, possibly, he had not the courage of his convictions. Lipman's work included an examination of the effects both of withholding and of adding artificial nitrogen to the mixed vegetation; his experiments remain the only ones expressly designed to examine the effect of added nitrogen upon the uptake by graminaceous plants of nitrogenous substances derived from host legumes.

Lipman's first published (1910*a, b*) experiments were only qualitative, but they resulted in "a striking proof of the ability of oats to secure an adequate supply of nitrogen when growing together with field peas in a soil devoid of nitrogen" (1910*b*). The method used was the simple one of growing the two plants in sand; the species were separated by either a porous or a non-porous pot. When separated from the peas by a porous partition, oats grew better and became much greener than the oats in a glazed and presumably impermeable pot. Since the effects were marked before the plants were ten weeks old, nitrogen derived from root decomposition would seem to be practically excluded from consideration. Lipman worked with numerous other mixtures, both in pots and in the field. It is curious to note how all the schools of subsequent workers upon associated growth have been unaware of previous work at the time of commencing their own. Lipman's work having escaped their notice, Lyon and Bizzell published in 1911 a paper entitled "A heretofore unnoticed benefit from the growth of legumes." Lyon and Bizzell's field experiments upon which their paper was based led to the same broad conclusion as Lipman had already drawn. Lyon and Bizzell's analyses showed that in the field a non-legume could attain a higher protein content when grown with a legume than when grown alone. (For a polemic regarding the question of priority, see Lyon and Bizzell (1913*b, c*) and Lipman (1913).) Lyon and Bizzell concluded that the increased protein content of a graminaceous plant grown in

association with a legume was due to nitrification promoted by the presence of the legume. Another paper by the same authors (1913*a*) discusses the query whether there is a mutual stimulation of plants through root influence. Though a number of field experiments on mixed vegetation of grassland were performed (good examples are the work of Evans (1916) and of Skinner and Noll (1919) (nitrogenous manuring)) no further work on the mode of uptake of nitrogen by one plant from another can be traced until 1926, when Stallings published a paper on "The form of legume nitrogen assimilated by non-legumes when grown in association." Stallings wrote: "That non-legumes, when grown in association with inoculated legumes under favourable conditions, profit by the association is a well-established fact," but, in his paper no bibliographical reference to the question was given. He worked with the curiously unpractical association of wheat and soya beans, grown separately as well as together. Twenty-four tables laboriously give a picture of his analytical results. The recorded crop weights and nitrogen contents suggest that the crop and absolute nitrogen yields were depressed or at best not increased, by associating the two plants, but this appearance may be due to the involved method of presentation. However, Stallings was not concerned with the crop yields, but with elucidating the nature of the substance presumed to have been transferred from soya to wheat. He concluded that "the beneficial influence exerted upon wheat by the inoculated soybeans was evidently due to soluble nitrogen, possibly ammonia, placed at the disposal of the latter by the former, when grown in association." Stallings's conclusions were vague, but he deserved credit for being the first to attempt to determine the form of nitrogenous compound excreted and taken up.

Comparing the nitrogen nutrition of red clover and white clover supplied with effective nodule bacteria, and with that of the same species supplied with ammonium nitrate under sterile conditions, Virtanen (1928, 1929) concluded that these species differed in their ability to make use of the nitrogenous compounds originating in the nodules. He was thus led to try with von Hausen (1931) the effect of various amino-acids, as well as inorganic compounds of nitrogen, as sole source of nitrogen for these plants. In 1927 Virtanen and von Hausen (1931) had noticed that when inoculated red clover was grown alone in sand, notable amounts of nitrogenous material were to be found in the sand. An excretion began at a very early stage of growth and was also observed to take place with vetches in water culture (Virtanen and von Hausen, 1931). These ingenious researches needed but three steps to complete them, namely, to show that non-legumes growing alone were able to utilise amino-acids; that amino-acids were excreted by leguminous plants, and that non-legumes could profitably utilise those excretions of legumes. All of these steps were made. X

Virtanen's first experiment on associated growth was performed in 1927 and reported in 1928 and 1929. It consisted simply of a demonstration that in absence of added nitrogen, oats grew sturdily when associated in a sand culture with inoculated peas, but failed to make any growth when the peas were not inoculated.

The next experiments by Virtanen and von Hausen (1930, 1931) were made upon red clover and meadow foxtail grown in association. These authors were the

first to introduce the notion of an effect of the proportion of non-legumes to legumes, though Lipman had already (1912) thought it worth while to record the numbers of each. These experiments with clover and meadow foxtail were performed in the successive years 1928 and 1929 with the species in the ratio of 1 and 2 grass plants for each leguminous plant. Both species and both sets of experiments showed a curiously wide variation in yields in the two years. No parallel pot and no control was set up. The experiments were made with sets of four pots, each pot being maintained at pH 5.0, 5.5, 6.0 or 6.5. This examination of the effect of varying degrees of acidity was another new feature of the investigations of associated growth. It was a logical consequence of earlier work upon varying the pH of growth of cultures of isolated bacteria; this in turn led to a comparison of the results of growing single species of leguminous plants: (a) in conjunction with their own nodule bacteria, and (b) aseptically fed with mineral nitrogen. The pH that was optimal for one species of the vegetation would not in general be optimal for another species. The maintenance of a range of acidities (cf. Wood, 1933) was desirable for a thorough examination of the effectiveness of any transfer of nutrients—if indeed, transfer be the word for so passive an uptake.

In addition to the red clover-meadow foxtail mixture, Virtanen and von Hausen (1930, 1931) investigated the growth of mixtures of peas and oats in the ratio 1, 2, 2.75, 4 and 5 oat plants per pea. These proportions were grown in pots at pH 6.5, which was optimal for the peas and also for the variety of oats (*Argus-Hafer*) employed. A further experiment with oats and peas grown in equal numbers at pH 5.5, which is unfavourable to the legume, but well tolerated by oats, showed that at that acidity the oats could still obtain considerable quantities of nitrogen from the peas. The authors also grew white clover with cocksfoot, and a composite mixture consisting of red, white and alsike clovers and timothy, meadow foxtail, cocksfoot, and *Poa* sp.

In all the Finnish experiments so far described, except in the red clover-meadow foxtail mixtures subjected to adverse conditions, favourable growth of the graminaceous constituents of the mixture was observed. The growth of the non-legumes was in fact of the same vigour as if an adequate supply of nitrogenous manure had been given. Nevertheless, there was evidence that when the ratios of the numbers of non-legume plants to leguminous plants approached or exceeded 2 : 1, the growth of both species suffered; in the case of five mixtures of peas and oats the set-back to growth was even more evident in the peas when the cereal was numerically preponderant.

The remaining pot and field experiments by Virtanen and his school, upon the question of associated growth, confirm the findings already given, and need little comment (see Table II below). General conclusions have been summarised by Virtanen (1933 *b*), Vartiavaara (1933), and in *Nature* (Anon. 1933). Mention must be made of the work of Virtanen (1933 *b*) on growing plants individually with their roots in sterile "closed systems" (plugged flasks containing sand and nutrients). As stated above he was able to show that under such conditions, red clover (*Trifolium pratense*) profitably utilised aspartic acid and the products of hydrolytic cleavage of

casein, whereas white clover (*T. repens*) was better nourished from ammonium nitrate. Inoculated peas excreted nitrogen in the following proportions (Virtanen, von Hausen and Karström, 1933):

Nitrogen as	% of total nitrogen
Amino-N	77.4
Ammonia-N	0
Amide-N	3.30
Volatile basic N	2.73
Melanin (humus) N	2.05

No results have been published concerning the ability of oats in single culture to take up amino-compounds. Peas, barley and wheat made use to varying extents of hydrolysed casein and of six single compounds of mineral and amino-acid nitrogen. Aspartic acid, and asparagine to a less marked degree, were claimed to be suitable for the nitrogenous nutrition of leguminous plants, at least during the first 5-7 weeks of life. Barley and wheat better utilised the nitrogen of potassium nitrate during a similar period. Such experiments upon the nitrogen nutrition of single species are not new in conception (*e.g.* Nakamura, 1894-7): these results are recorded here solely on account of their relation to the problem of associated growth. Evidently, more work requires to be done upon the question of the forms of nitrogen metabolised and excreted by legumes and assimilated by non-legumes grown in association with them. Virtanen (1933 *b*) has suggested that plants can obtain part of their anabolic carbon from nitrogenous compounds of that element.

Virtanen, von Hausen and Karström (1933) grew one leguminous and one oat plant together in flasks maintained under conditions sterile except for the presence of specific legume nodule bacteria. The plants were sometimes entirely within the glass vessel. An interesting variation of this technique was the growth of peas and oats in three-necked Woulff's bottles; each aseptic bottle contained the roots of one inoculated pea plant and one oat plant growing in sand, while the aerial part of each plant emerged through one of the necks. This latter arrangement permitted a freer development of the plants. In all such experiments, the inoculated legume successfully acted as nitrogen foster-mother to the oat.

The alder, though not a legume, bears upon its roots nodules produced by nitrogen-fixing bacteria. Virtanen and Saastamoinen (1933) have shown that inoculated alder plants excrete nitrogenous substances into sand in a pot culture, whereas an uninoculated plant did not excrete an appreciable amount of nitrogen. Inoculated alders made better growth than alders not inoculated but supplied with ammonium nitrate as sole source of nitrogen.

The reality of the production of nitrogen compounds by the roots of one plant, in forms in which they are available for the nutrition of another plant, can now be considered as established, though the mode of excretion is still obscure and many possible factors have not yet been tested. In 1931 Thornton and Nicol (1934) set up a pot experiment in sand, intending to examine some aspects of nitrogenous manuring upon competition between a legume and a non-legume. Nitrogen as

sodium nitrate was added at three levels of manuring but in one dose to each of a number of parallel pots, to which basal nutrients were also given. Lucerne (var. Grimm: *Medicago sativa* \times *M. falcata*) and Italian rye grass were then sown and the seedlings were thinned out to leave equal numbers of plants of each species. Lucerne was also grown alone under the same conditions. The lucerne seed was inoculated with an efficient strain of lucerne nodule bacteria. Three parallel pots of each treatment were removed when the plants were 2, 3, 4 and 5 months old, and the plants were harvested so as to secure both roots and tops of each kind separately. These successive reapings enabled the course of growth to be represented graphically, and in this respect this experiment is unique among experiments on

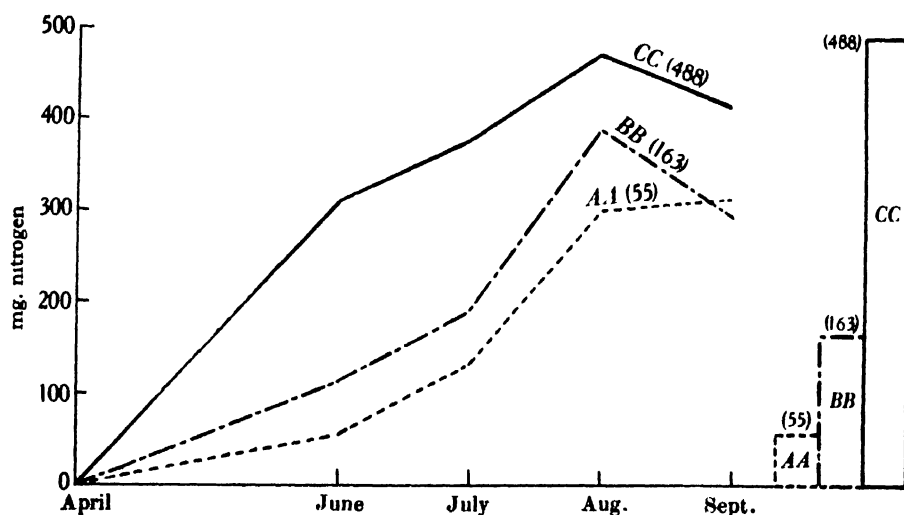


Fig. 1. Milligrams of nitrogen in tops and roots together in Italian rye grass grown with lucerne at three levels of nitrogenous manuring. The columns at the side represent on the same vertical scale the amounts of nitrogen (as sodium nitrate) added in one dose at the commencement of the experiment.

associated growth. In the pots that had received the two lowest dressings of sodium nitrate, the grass had a content of nitrogen equal to several times the amount supplied in the manure. Fig. 1 shows the nitrogen contents in the grass (whole plants). The content of nitrogen in the grass growing in those pots that received the largest dressing of sodium nitrate closely approached a figure which might have suggested 100 per cent. recovery of the added nitrogen. It is nevertheless impossible to say how much of the grass nitrogen was derived from the sodium nitrate, and how much from the lucerne. It is evident that when growth of the lucerne was not depressed by nitrogenous manuring, the grass contained nitrogen which could have come only from the legume. Even in the most highly manured series, the grass had probably derived some nitrogen from the lucerne.

(b) *Uptake of phosphorus.*

Though numerous experiments on root excretions have been made, only one has been found with a bearing upon the subject of associated growth. Domontovich, Shestakov, and Polossin (1933) found that oats in association with lupins took up much more phosphorus from phosphate rock than when grown alone. In this association the effect upon the non-legume of the legume's root excretions was indirect; it was due to the lupins dissolving more phosphate than they required. It does not appear to have been determined whether nitrogenous compounds are concerned in the solubilising effects. Probably other examples of this type of association will be revealed; cf. Prianishnikov (1934). Tyagny-Ryadno (1933) has described such a solvent action upon phosphorite, due to *B. mycoides*.

(2) ACCESSORY FACTORS AND THE GROWTH OF PLANTS; A SUGGESTED RÔLE FOR LEGUMINOUS PLANTS.

Many agriculturists are beginning to think that accessory factors are somehow concerned in the nutrition of plants. The accessory substances (vitamins, auximones, growth and co-growth substances, phytamins) are not necessarily compounds of nitrogen, but the question of their production is relevant to problems of manuring and will be briefly discussed. There are several aspects which it is essential to distinguish. These are summarised below.

Sources of possible accessory factors in plants, whether required for the proper growth of the plants themselves or for animals which ingest them.

- (1) Derived from inorganic compounds of nitrogen:
 - (a) Directly built up by and within the plant.
 - (b) Synthesised by micro-organisms living free in the soil.
- (2) Derived from organic sources of nitrogen:
 - (a) Synthesised by nodule bacteria:
 - (α) In symbiosis with their host plant and used wholly within that plant.
 - (β) In symbiosis with their host plant, but taken up by associated plants.
 - (b) Found in organic manures derived from land or marine plants or animals:
 - (α) Already formed therein (i) in organs and tissues, (ii) by biological activity in the gut of animals.
 - (β) Formed by the action of micro-organisms (including free-living nitrogen-fixing organisms) thereupon.

Hitherto, experimental investigation has been almost exclusively devoted to the vitamins (required by animals) produced in germinating and mature plants. The production of vitamins in plants in cultures has been extensively investigated—by the school of Virtanen, amongst others. It should be borne in mind that vitamins, however essential for animal growth, may be a waste product in plants. Virtanen (1933 (b)) was tempted to conclude that the production of vitamin C and of the pro-

vitamin, carotin, was correlated with the growth of the plant, and that these substances are growth factors of plants. This tentative conclusion would suggest that the plants investigated were "raising themselves by their own boot-straps." It is reasonable to suppose that the vitamins needed by the animal may not be identical with the accessory substances required for the plant growth. It is therefore convenient to distinguish accessory plant-growth substances as phytamins. Evidence is accumulating respecting their reality.

Viswa Nath (1932) and his co-workers at Coimbatore and elsewhere have taken a prominent part in investigating the influence of manuring upon vitality of plants (Viswa Nath and Suryanarayana, 1927). Indian cattle manure had a high value in this respect, but the unsaponifiable matter of cod-liver oil, as well as farmyard manure extract, stimulated growth and reproduction of plants. Hartley and Greenwood (1933) in Nigeria, and Tyagny-Ryadno (1933) in Russia, have recorded effects from small amounts of farmyard manure greater than could be accounted for from consideration of its content of common nutrient elements alone. The extent to which vitamins are produced by rational nitrogenous manuring of crops is of sociological importance and deserves attention (Armstrong, 1933). This aspect of crop manuring has a bearing upon the ability of animals to resist disease (McCarrison, 1926; Howard, 1933). Hartelius (1933) has reviewed the literature on the "growth substance B" that occurs in urine and stimulates the growth of plants. Extracts of root, stem and growing tip, of seedlings of maize three or four days old were found by Popoff (1933) to stimulate the growth of *Euglena gracilis*; this may have been due to vitamins, in which young plants are rich, or to amino-acids. Thornton and Smith (1914) noted a marked stimulation of *Euglena* by presence of amino-acids, especially tyrosine in aqueous solution. Mockeridge (1920) thought that true plant-growth accessory substances were nucleic acids or their constituent nitrogenous bases. An extensive field of work appears to be open.

It is not known to what degree plants rely upon animal excretory products. Results from Agdell Field, Rothamsted, show that the ratio of the yield of barley on the "clover" side to that on the "fallow" side (without clover) appears to be altering. No dung has been carted on to these plots since about 1844, but, for a time, sheep were fed on the turnip crops upon the field. The last record of animals on the field was made in 1876. The suggestion that some of the crops may be suffering from an insufficiency of some substance contained in dung is supported by consideration of the "continuous clover" plot, which was laid out in richly manured garden soil in 1854. Since about 1874, increasing difficulty has been experienced in getting the clover to grow and maintain itself on this plot.

The poor quality of continuously grown cereals shows that dung alone is not enough. There remains to be ascertained how much the benefits of sequence, as well as association, of plants are referable to accessory substances (phytamins) produced by members of the association. It may well be that nodule-bearing plants have a peculiar aptitude for the production and bequeathing of phytamins. Residual effects from leguminous crops, especially lucerne (Nicol, 1933), have been observed to persist for many years. It is possible that the manurial value of legumes, as well

as of animal manures, is not entirely due to their leaving a residue of mineral nutrients.

If these notions can be shown to be well founded, new light may be thrown not merely on the problem of mixed vegetation, but also upon the value of rotation of crops in the field and upon the succession of plants in nature.

(3) ASSOCIATED GROWTH IN PRACTICE.

(a) "*Competition*" between legumes and non-legumes.

Of great practical importance to agronomy has been the recent introduction to grass mixtures of seed of wild white clover, a perennial. Clovers had long been incorporated in "seeds" (see p. 401), but the introduction of "wild white" gave such remarkable results in the improvement of grassland that it ranks as a fresh step. It had been noticed that certain Kentish pastures wherein wild white clover was abundant had an exceptionally high feeding value. Seed of the clover was saved and distributed, and now the "wild white" is cultivated—though "original Kentish" still fetches the highest prices. In other countries advances have also been made in respect of the leguminous component of herbage: e.g. in Austria (Jentsch, 1927); in Australia "subterranean clover" (*Trifolium subterraneum*) has been extensively introduced, while New Zealand produces an export surplus of wild white clover seed. In three decades or so the value of wild white clover has been so fully recognised that it may be said to be invariably a component of the best seed mixtures used for establishing permanent grass.

The period of introduction of "wild white" coincided closely with the period of development of new nitrogenous fertilisers intensively marketed. Many of them, as well as the older preparations, were used on both newly sown and on old clovery grassland, but the results obtained were often equivocal. So long as evidence of the bulk yield of crop was accepted, small progress was made concerning the real effect of nitrogenous manures upon swards, but when exact botanical and chemical investigations of the manured crop were multiplied it came to be recognised that nitrogenous manures were seldom of benefit (Brown, 1932*a, b*). In one of the earliest publications of Lawes and Gilbert (1858) the statement was made that "increased growth of the *Leguminous herbage of the meadow* was not favoured by the direct supply of nitrogenous manures," but their observations remained almost isolated until comparatively recently. Now the substance of the above-quoted remark of Lawes and Gilbert is accepted practically everywhere. Workers on the manuring of grassland in many countries have come to the same almost unanimous conclusion: when legumes are a component of a mixed vegetation, an addition of combined nitrogen is of little or no benefit in increasing the nutritive value of the crop.

The form of nitrogenous compound or compounds built up by the nodule bacteria cannot be said to be known with certainty, but it is probably an organic compound. Leguminous plants are nevertheless capable of having the nitrogen gained from their nodule bacteria superseded or replaced by externally applied

nitrogen. The nodule bacteria may be entirely lacking, under either natural or experimental conditions, or the activity and numbers of the nodules may be suppressed by the action of large doses of nitrate: in any such event, added nitrate, or ammonium salts, will enable leguminous plants to function as non-legumes, and take up inorganic nitrogen. It is not usually economic to allow bought nitrogen to supersede the natural gratuitous building up, by the nodule bacteria, of atmospheric nitrogen into plant tissue.

The benefit derived by non-legumes from nitrogenous manures is thoroughly established. It seems curious, therefore, that an addition of combined nitrogen to a mixture of the two classes of plants, each of them separately able to utilise the manure, should result in no apparent gain of harvested crop or in the number of animals fed off a given piece of land. (The still recent problem of "rotational grazing" is almost as much a matter of animal husbandry as of agronomy and deserves separate treatment; cf. Jones (1934).) This widely recorded failure of mixed vegetation treated with artificial nitrogenous manures to increase the yield of protein and of nitrogen recovered in the crop is well instanced by the results of field experiments at Rothamsted, of which two may be quoted here. A forage mixture experiment (oats or barley, with vetches or peas, and with a basal sowing of field beans) was commenced on arable land in 1930, ammonium sulphate being used as nitrogenous manure with a full basal dressing of phosphatic and potassic manures. In its first year the following results were obtained (all mixtures):

Nitrogen added (cwt. per acre)	0	0.2	0.4
Yields of dry matter (cwt. per acre)	23.3	31.8	35.8
Percentage of crude protein in crop	11.7	9.6	8.6
Nitrogen in the crop (cwt. per acre)	0.42	0.44	0.44

The bulk of the crop was increased, so that an uncritical estimate by eye, or by weight alone, would have produced testimony to the virtue of the nitrogenous manure. It can be seen at a glance, however, that no significant proportion of the applied nitrogen was recovered in the crop. A similar result was obtained in 1931, when nitrogen in the forms of ammonium sulphate and sodium nitrate were supplied to combinations of cereals and legumes. (See *Rothamsted Reports* for 1930, 1931, and 1932, and Tables II and III below.)

In 1932 another field experiment was set up, using oats and vetches only, in several proportions. Some results of this experiment are recorded diagrammatically in Fig. 2.

Differences between the different seeding rates are significant, but the total nitrogen contents of the crops were not appreciably altered by the application of nitrogen (as ammonium sulphate). The total nitrogen content was maximal with both treatments for a mixture of 50 lb. oats and 150 lb. vetches per acre, and was absolutely highest when no nitrogenous manure was applied to a mixture of that composition.

In the lucerne and grass (pot) experiment by Thornton and Nicol (1934) sodium nitrate depressed the growth of a mixture of lucerne and grass. In those

pots which had received most nitrate, the lucerne, in association with grass, grew much less strongly than in the less highly manured pots (Fig. 3). It is therefore fair to draw the inference that the more highly nitrogenously manured lucerne plants had less nitrogen to place at the disposal of the grass. The nitrate increased the growth of the grass (at least the two highest doses did) and the factor of root competition entered in. The addition of nitrogen as sodium nitrate to this particular association progressively reduced the total nitrogen content of the mixtures and was cumulatively adverse to the leguminous partner.

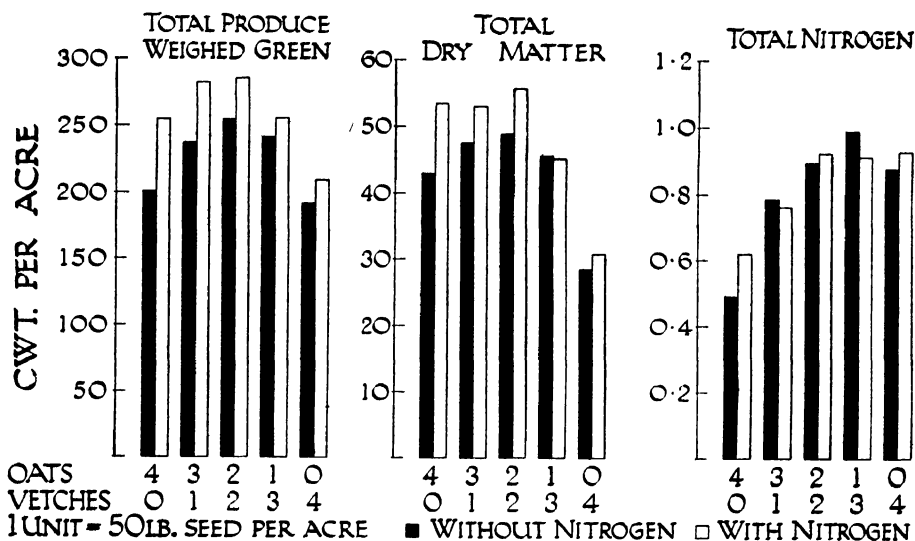


Fig. 2. Fodder mixtures of oats and vetches, Rothamsted.

On grassland the almost invariable effect of nitrogenous manures applied to mixed vegetation is to repress the nitrogen-richer legumes and assist the non-legumes. Only two explanations are apparent: either (a) combined nitrogen is toxic to leguminous plants, or (b) competition between the two sorts of plants results harmfully to the legumes when combined nitrogen is artificially supplied to the mixture.

The question of toxicity need not detain us. Ammonium sulphate has long been used to "kill out" clovers on lawns. It may be that its action is due to its strengthening the grasses, and it was also thought that ammonium sulphate, being "physiologically acid," made the soil acid to a point when the clovers languished. Blackman (1932) who worked with ammonium compounds—the sulphate, and also the phosphate, which is not so "physiologically acid" as the sulphate—found that a perceptibly toxic action of the ammonium radicle upon weeds, though not upon clovers, manifested itself before the soil had become acid.

A possible explanation of the observed facts lies in the effects of "competition"—the reaction of the members of the plant population upon one another. The extent

acts upon its neighbours has necessarily been obtained with pot cultures, maintained sometimes under conditions of strictly controlled sterility. Such controlled and unnatural methods, it may be recalled, were the only ones of value in deciding the question whether symbiosis existed between the leguminous plants and their nodule bacteria.

Applied to the study of the problem of associated growth, both laboratory and field experiments have demonstrated that the "competition" of legumes and non-legumes can be made to assume a considerable value to man. With the help of the information furnished by such experiments, man himself can benefit from the association of these plants. Associative growth is in fact growth in equilibrium, and only when this equilibrium is destroyed does the competition factor become noteworthy.

(b) *Cover crops. The value of leguminous crops in a rotation.*

Sometimes the term "cover crop" is applied to an intercrop in an orchard, or to temporary and subsidiary crops intended to cover the ground before a ploughing-in as green manure. In British practice, and in the only sense in which they will be discussed here, cover or "nurse" crops consist of a single species, usually a cereal, amongst which another and usually leguminous crop is sown. Use of the term "nurse" crop for the cereal seems unfortunate, since the cereal tends to rob rather than to protect the undersown legumes.

Lipman's review (1912) of some historical references to the practice of sowing legumes in cover crops is sufficient to indicate the extensiveness of observations made thereupon. Jentsch (1927) has given an account of the little-known "Egartwirtschaft" practised over the greater part of southern Austria; this rotational system is based on undersowing with clovers.

In Britain, however, very few experiments have been made for the purpose of determining the mutual actions of a cover crop and its undersown crop. This is surprising in view of the agricultural importance of the practice of sowing clover in barley, for example. The still controversial results of the Duke of Bedford and S. U. Pickering (1919) deserve mention, but they cannot be briefly discussed.

With a cereal and a legume, the effects of competition are recognised in practical agriculture to the extent of advising that if a cover crop is used at all, the sowing of non-legumes should be thin to give the legume a good start when the legume is to be the principal crop, as in the case of lucerne sown under a cereal. Commonly the cereal is intended to be the principal crop, so that a normal sowing of the cereal is made, and the usual nitrogenous fertilisers are applied. In such cases the legumes are clovers sown either singly or as a mixture of clovers and grasses. This undersown crop (the so-called "seeds" ley) is intended for cropping in the year following the cereal, and the effects of competition upon the legumes are tolerated during the year of sowing. These effects are due to dense sowing and nitrogenous manuring of the cereal, as well as to restriction of light by the maturing cereal crop.

Undersowing a leguminous crop in a cereal differs from growing a fodder crop, inasmuch as the undersown crop is not intended to be harvested with the cereal.

Numerous field trials made by Thornton (1929*a*) showed that inoculated lucerne was better able than uninoculated to make good growth under a cereal cover crop even where some wild nodule bacteria were present. Supplying active nodule bacteria by "inoculating" the seed gave the lucerne a good start, and this produced a lasting effect upon the growth of the lucerne. Since the only comparisons made were between lucerne inoculated and uninoculated, sown bare and under a cereal, no crop yields of the cereals are available. Agdell Field four-course rotation, Rothamsted, offers one of the few examples of barley sown both bare and undersown with clover. The growth of the undersown barley is perceptibly better during its early stages, and on the two pairs of manured plots (with and without artificial nitrogen) the average yield of barley is higher where it has been undersown. The influence of the general level of nutrition is clearly shown on this field by the poor growth of both barley and clover where no manure is supplied.

Rothamsted experiments provide quantitative information every four years from Agdell Field regarding the effect of clover upon the yield of the cover crop. None of the modern four- and six-course rotation experiments at Rothamsted and Woburn has yet completed a cycle. A one-year's experiment was carried out at Rothamsted in 1931 to compare the effects of no undersown crop, pure clover, pure rye grass, and a mixture of clover and rye grass, all with and without added nitrogen as sulphate of ammonia. The yields of barley tended to be increased by leguminous undersowings (Rothamsted, 1931; Russell and Bishop, 1933).

An extensive search has revealed only one other record of the effect of undersowing legumes in a cover crop. Experiments were performed annually by the Statens Planteavlssudvalg (1917) from 1910 to 1917, with spring oats (in 1910-11 oats mixed with barley) undersown with Italian red clover and with serradella. Nitrogenous manure was applied but no dung. Some results are given in Table I.

Table I. *Yields of cereal grain in kg. per hectare, Statens Planteavlssudvalg, Denmark.*

	Grown alone	Undersown with	
		Serradella	Red clover
Mean yields 1910-16	1004	1052	1062
Yield in wet year 1916	1768	2272	1936

Most agricultural teachers and researchers upon the effect of undersown legumes upon crops (*e.g.* clover, Shutt, 1898; sweet clover (*Melilotus* spp.), Crosby and Kephart, 1931) have devoted their attention to residual manurial effects, ignoring the effect of undersown legumes upon the "nurse" crop. A preponderating manurial action has been ascribed to nitrogen in the dung of the classical four-course rotation. It has not been widely realised that effects of the legume break are traceable during three years out of the four, and that nitrogen (as dung) is carted on to the field only in that year in which sufficient nitrogen manuring from the growth of legumes is not available.

III. TABLES SUMMARISING EXPERIMENTS ON ASSOCIATED GROWTH.

Table II summarises most of the experiments (other than Lipman's and those upon grassland) known to the author which record the effect of growing non-legumes together with a host plant in the absence of added nitrogen. Lyon and

Table II. *Experiments, other than Lipman's, on associated growth, without added nitrogen.*

Made: (a) in U.S.A.; (b) in Finland; (c) at Rothamsted, England.

Season of growth	Host plant	Receptor (non-legume)	Numbers of non-legume plants to 1 legume in mixtures	A* Field P Sandpot S Flask	Authors	Year of publication
1910	Peas	Oats	0.8	A	Lyon and Bizzell (a)	1911
1910	Red clover	Timothy	—	A	Do.	1911
1910	Lucerne	Timothy	—	A	Do.	1911
1924	Soya	Wheat	1.5	P†	Stallings (a)	1926
1927	Peas	Oats	—	P	Virtanen (b)	1928, 1929
1928 and 1929	Red clover	Meadow foxtail	1 and 2	P	Virtanen and v. Hausen (b)	1930, 1931
1929	Peas	Oats	1-5	P	Do.	1930, 1931
1930	Peas	Oats	1	P†	Do.	1930, 1931
1932	Vetches	Oats	0.33, 1, 3	A	Rothamsted Exp. Sta. (c) (see also Fig. 2 and Table III)	1933
1932	Peas	Oats	0.33	A	Virtanen (b) (Finn. Biochem. Inst.)	1933 a
1932	Peas	Oats	0.41	A	Virtanen (b) (Central Finn. Agric. Org.)	1933 a
	Red clover	Timothy	—	A	Do.	1933 a
1931	Peas	Oats	0.17-4	A§	Vartiovaara (b)	1933
1932	Peas	Oats (2 var.)	—	A	Do.	1933
1932	Peas	Oats	1	S	Virtanen, v. Hausen and Karstrom (b)	1933
1933	Alder	Birch or pine	—	—	Virtanen and Saastamoinen (b)	1933

* In field experiments (A) the ratios given are not based upon the number of plants but are ratios of weights of seed sown.

† His proportions are not clearly stated in the original.

‡ Experiments at a lower pH than in the preceding.

§ Graphs were constructed giving as abscissae these proportions by weight of seed sown, although the recorded proportions of seedlings varied from 0.9 to 11.2.

Bizzell published no statement regarding manuring of their experiments. Pilz (1911) wrote a pioneer paper of wide scope, which cannot be discussed in this review. Especially brilliant examples of the effects of legumes in mixed vegetation were given by La Flize (1892). (His cordial mention of the work of Petermann apparently refers only to the mode of distribution of manures in soil (Petermann, 1884).)

Other work omitted is that of Bagge (1927), itself a summary of Danish field experiments, and Kellerman and Wright (1914)—a report upon various mixtures grown on nineteen different soils. These authors contended, as did Evans (1916), that considerable benefit resulted, to the non-legume at least, when plants were grown in association. The qualitative observations of Karraker (1925) are of interest.

Ellett, Hill and Harris (1915) thought that the question of benefit from association was open. They are the only investigators to work with plants in greenhouse beds. McClelland (1928), who worked in the dry conditions of Arkansas, suggested that the combination in the field of soya beans or cowpeas with maize was undesirable. It is a common practice to sow such an association in parts of the United States, but McClelland concluded that a true competition for a restricted moisture supply was set up, which was harmful to the growth of maize.

Mooers (1927) stated that in Tennessee soils not moisture but nitrogen was the limiting factor; when cowpeas or soya beans were planted with maize, the yield of maize was depressed. The mixed vegetation gave a larger fodder crop of grain than either crop did if grown singly. The depression of maize yield may nevertheless have been due to competition for moisture on account of the different root habits of the plants. In all of Lipman's (1912) mixtures of maize with cowpeas or soya

Table III. *Experiments on associated growth, with added nitrogen.*

All performed at Rothamsted, England.

Season of growth	Ratios of higher to lowest dressing of mineral nitrogen N/S or S/Amm.†	Legume host plant	Receptor (non-legume)	Numbers of non-legume plants to 1 legume in mixtures	A* Field or P Sand pot	Authors	Year of publication
1930	1, 2, S/Amm.	Peas	Oats	0.58	A	Rothamsted Exp. Sta.	1931
		Vetches	Oats	0.58	A	Do.	
		Peas	Barley	0.80	A	Do.	
		Vetches	Barley	0.80	A	Do.	
1931	1, 3.3, 10, N/S	Lucerne	Italian rye grass	1	P	Thornton and Nicol	1934
1931	1, N/S v. 1, S/Amm.‡	Peas	Oats	0.95	A	Rothamsted Exp. Sta.	1932
		Vetches	Oats	0.95	A	Do.	
		Peas	Wheat	0.95	A	Do.	
		Vetches	Wheat	0.95	A	Do.	
1932	1, S/Amm.	Vetches	Oats	0.33, 1, 3	A	Do. (see Fig. 2)	1933

* In field experiments (A) the ratios given are not based upon the number of plants but are ratios of weights of seed sown.

† N/S = nitrate of soda; S/Amm. = sulphate of ammonia.

‡ With basal manuring with "Adco" compost.

beans, growth of the maize was depressed (cf. Zavitz, 1927). Kaserer (1911) has recorded root interpenetration in mixed crops.

The records of the Cawnpore (1906) experiments, which presumably represent those suggested by Leather (1897), are not informative enough to make discussion profitable.

It will be seen from Table II that Virtanen and his co-workers intend to study the effect of commensalism between two non-legumes, namely, the nodule-bearing *Alnus* and a forest tree. The work of de Peralta and Estioko (1923), on the effects upon rice of the drainage waters from other monocotyledonous plants, should be mentioned.

Table III summarises such experiments upon mixed vegetation (other than grassland) grown with added nitrogenous manures, which provide reliable information upon the transfer of nitrogen from legume to non-legume. None of these was designed to investigate the question of transfer, but they have a bearing upon it. In Denmark, investigations upon the growing of mixed fodder crops have been made since 1899 at least (Anon. 1909). The Rothamsted field experiments all include a comparison of nitrogen with no nitrogen. For other pot experiments with nitrogenous manures added to mixed vegetation see Joulie (1886); Nobbe and Richter (1902); Remy and Vasters (1931), and a graph based upon the results of the last (Thornton and Nicol, 1934). A summary of some work on the subject of associated growth is given by Fred, Baldwin, and McCoy (1932).

IV. CONCLUSION.

It was noted by Raymond Pearl that biologists have been relatively slow to appreciate population factors in the study of living organisms. A single animal has been taken by zoologists to be typical of a species. Bacteriologists give weight to the colonial behaviour of a micro-organism which they are describing, but they study the organism in pure culture, isolated from its fellows, even though it has originated in such a biological complex as soil. Agricultural botanists have been prone to regard competition as something undesirable; probably because they have been mercenarily concerned with the suppression of weeds in otherwise pure cultures of cash crops. In grassland, competition of grasses has been regarded as a nuisance because it is usual for the botanical analysis of a sown sward not to correspond with the percentage composition of the seed mixture sown. These examples no doubt have an anthropocentric basis. Acknowledged authorities have been slow to perceive beneficial effects resulting from "competition." Sir John Lawes was an acute observer, and he was especially interested in the problems of nitrogen nutrition of plants, yet, in his and in Sir Henry Gilbert's published work there is little evidence of any belief that one plant might take up nitrogen from another. Munro and Beaven (1900) had access to Rothamsted records and observations, but in their survey of the effects of clover upon barley in rotation, they imputed such effects to previous, and not to concurrent, leguminous crops. No reference to associated growth has been found in Russell (1932); and benefits

from associated growth were substantially ignored by Clements, Weaver, and Hanson (1929).

Use of the word "competition" is unfortunate, owing to its suggestion of rivalry. It has been shown that activity and efficiency of bacteria in mixed culture with other bacteria (Mahmoud Selim, 1930) and Protozoa (Nasir, 1923; Cutler and Bal, 1926; Meiklejohn, 1930, 1932; Telegdy-Kováts, 1932) are greater than in pure cultures. This is probably true in the soil also. In the case of mixed vegetation just discussed the "competition" between legumes and grasses is essentially a symbiosis. It is, moreover, a double symbiosis in that cereals and grasses profit directly by atmospheric nitrogen fixed by a symbiosis between the leguminous plant and its nodule bacteria. The conditions of a mixed vegetation resemble the case of an ecto-parasite living harmoniously upon a host which itself is nourished through the agency of mycorrhiza. To many, the conception of grassland and of mixed forage crops suggested by this parallel will be new. A true competitive rivalry supervenes only through the agency of man—giving an excess of artificial nitrogen, or by overstocking with grazing animals. The growth of leguminous plants at the expense of non-legumes is to some extent favoured by manuring with carbohydrate, or carbohydrate-rich material (Murray, 1921; Thornton, 1929*b*), but this is probably an effect due to removal of combined nitrogen. Based upon a concept of a population existing not in rivalry, but in harmony, the soundest method of manuring mixed vegetation is to supply abundant phosphate and potash, with lime if necessary, and by thus sustaining and increasing the vigour of the leguminous component, to encourage that double association upon which the natural well-being of the floral population depends.

V. SUMMARY.

About 1840, the beginning of the era of scientific agricultural chemistry, many chemists believed that ammonia was the principal, if not the sole form in which nitrogen was taken up by all plants. This view was abandoned, and towards the end of last century it was generally believed that with the possible exception of the Leguminosae, the higher plants took up their nitrogen almost solely from nitrate. This belief was in large measure founded upon the results of excessive attention paid to the conditions of soil which was not bearing vegetation.

The discovery that leguminous plants were able by the help of specific bacteria to utilise atmospheric nitrogen was not thought to extend to any of the other higher plants. Though widespread use had been made by practical farmers of leguminous plants in association with non-legumes, the idea of commensalism between legumes and non-legumes did not arise amongst agricultural scientists until the present century was well advanced. The acknowledged benefits attained through the growth of legumes were over long ascribed to nitrification of decayed roots resulting from some previous (not to a simultaneous) legume occupancy. This theory may have been correct for the conditions of single crops on arable soils, but was inadequate to account for the comparative failure of grassland and mixed forage crops to respond profitably to fertilising with quickly acting mineral nitrogenous manure.

The rôle of nitrate in the soil is not clearly understood; nitrate is most likely an end-product of micro-organic decomposition of organic materials. Its presence is detectable in notable amounts, and most clearly, in the absence of plants. This does not necessarily imply, as it was once thought, that it is preferentially absorbed by plants; it is suggested that plants can absorb some of the less highly oxidised forms of nitrogen which are the precursors of nitrate. In other words, the finding of nitrates in considerable amounts in soil indicates that there has been a local surplus of nitrifiable nitrogen compounds which plant roots have been unable to reach and consequently to absorb. No single compound of nitrogen can be named as the primary component of the nitrogenous nutrition of plants.

Evidence is presented that non-leguminous plants can profitably utilise compounds of nitrogen built up by the symbiotic life of nodule bacteria within their proper leguminous host plants. Some insight into the nature of the transferred compounds has been gained, though the conditions *in vitro* do not admit of facile extension to natural conditions. The mode of transfer from legume to non-legume is still obscure, but the existence of a transfer can be taken to be well established; it represents a stage in a double symbiosis of which the importance has not been fully appreciated. It is probable that in the nitrogenous nutrition of plants some factors are involved which are not yet formulated. These accessory factors may be found to derive ultimately from the animal, aided by activity of legume nodule bacteria in the soil.

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ADDENDA.

A paper by H. C. Trumble on "The agricultural features of *Phalaris tuberosa* and allied forms" (*Journ. of Agric. South Australia* (1933), **37**, 400) contains the novel postulate that for sustained productivity of *P. tuberosa* "a suitable associate legume" is desirable. A further paper by H. C. Trumble and J. G. Davies is announced, in which will be given "evidence of the value of legumes in association with permanent grasses." J. H. Gurski has compared oats-barley and oats-vetch mixtures. *Doświadc. Roln.* (1927), **3**, Parts (Części) III-IV, 55. Warszawa.

THE EXPERIMENTAL PRODUCTION OF MUTATIONS

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CONTENTS.

	PAGE
I. Introduction	411
II. A critique of older experiments	412
III. Criteria of the technique for experiments on the production of mutations	413
IV. Radiation genetics	414
(1) Historical attempts, and the first experiments of H. J. Muller	414
(2) General validity of the effects of short-wave radiations on the process of mutation	418
(3) Relation between the quantity of radiation and the mutation rate	419
(4) Quality of radiation as related to the process of mutation	422
(5) Various conditions which might have an influence on the induced process of mutation	424
(6) Types of induced mutations and comparison of induced and spontaneous processes of mutation	432
(7) The nature of the effect of rays on the process of mutation	438
(8) Applications and conclusions	440
V. Heat and other treatments as causes of mutation	446
(1) Temperature experiments	446
(2) Experiments with other agencies	449
VI. General conclusions	451
References	452

I. INTRODUCTION.

THE possibility of influencing, or even directing, the heritable variability of organisms is unquestionably one of the central problems of biology. And we know that biologists, and especially the evolutionists, were already interested in it at the end of the past century. But until recently this problem was unfortunately entangled in a morass of different more or less speculative evolutionary deductions. Even experimental work was influenced chiefly by the desire to prove or disprove the Lamarckian theory of the "inheritance of acquired characters"; in most cases it had already influenced the premises of the experiments in such a way that it was quite hopeless to draw any exact conclusions from the results obtained. This type of work will not be reviewed here. Only a short critique of it will be given, in order to show what kind of technical errors should be avoided in experiments on production of mutations.

Still another group of experiments will be omitted in the present review, as it has only an indirect connection with genetic problems: the histo- and cytopathological work done on treated germ cells. Since the first classical experiments of Gerassimow (1901), in which he succeeded in inducing the doubling of the set of chromosomes in *Spirogyra* by cold treatment of the cells, much work was done on the experimental influence of different agents (especially X-rays and radium) upon the nuclei of cells. But in almost all cases, except the recent work done by geneticists using suitable material, no genetic tests or evaluation of the induced chromosome variations were made. The most important results of this kind of work have already been summarised in another review (P. Hertwig, 1927).

Thus, within the limits of the present review there will only fall genetic experiments *sensu stricto* on the experimental production of heritable variations, performed on suitable objects and using exact technique. In the discussion of the principal questions, work on *Drosophila* will occupy a dominant position; not because the present author, being himself a *Drosophila* geneticist, is better acquainted with this material, but because of the actual conditions in experimental genetics, and of the fact that *Drosophila* has many advantages for exact genetic experiments.

II. A CRITIQUE OF OLDER EXPERIMENTS.

As has already been mentioned, we will not review in detail the older experiments dealing with the "inheritance of acquired characters," since they have already been reviewed several times (Semon, 1912; Kammerer, 1925; Wladimirsky, 1927). These experiments, if analysed critically, all show that no results were obtained at all. This is true even for the most recent experiments of this kind (Guyer and Smith, 1920; Dürken, 1923; Wladimirsky, 1929), performed at a time when the knowledge of modern genetics was already widely spread.

The reasons why all these experiments are of no scientific value are the following: (1) unsuitable material, (2) inexact technique, (3) small numbers of tested individuals and cultures, and (4) absence of exact genetic knowledge of the "normal" and "induced" variations in the tested objects. It is astonishing how obtusely all these experiments were planned and performed. The most inconvenient objects (*e.g.* salamanders or genetically quite unanalysed butterflies) and complicated or absolutely unanalysed reactions (*e.g.* formation of lens antibodies or formation of conditioned reflexes) were often used in this kind of experiment. The technique of treatment, as well as the breeding methods of the treated and control material, were in almost all cases insufficient to give sound results. Under the influence of the idea of "somatic induction" most of the authors did not concern themselves at all about the question of the penetration of the applied agent into the germ cells of the treated individuals. The breeding (genetic) methods and the numbers of tested individuals were such that, even in the cases with positive results of the treatment, the effect on mutability could not possibly be detected. And, last but not least, the genetic knowledge about the organisms taken as experimental objects was very poor: the cultures were not sufficiently inbred to secure pure and homogeneous material, the normal (spontaneous) rate and the kind of heritable variations

appearing in the material were quite unknown, and the characters said to be induced were not analysed genetically in an adequate way.

The chief theoretical error of almost all of these experiments was the attempt to solve directly certain evolutionary problems, without consideration of the many purely genetic (and sometimes even physical) questions and details, which must be analysed and solved in any experiment dealing with the artificial induction of genotypic variations, before any general conclusions can be drawn.

The older experiments have thus only a negative significance: they are a kind of warning, showing us the mistakes which we have to avoid, and the precautions that we have to take in planning and performing experiments on the induction of heritable variations.

III. CRITERIA OF THE TECHNIQUE FOR EXPERIMENTS ON THE PRODUCTION OF MUTATIONS.

In order to be able to reach conclusive results the following requirements must be fulfilled in performing any experiment on the production of heritable variations.

The *first requirement* is the genetic purity of the material used in the experiments. The stock from which the control and treated material is to be taken must be genetically analysed and closely inbred for several generations at least. Wild populations, as well as laboratory stocks kept for long periods in unanalysed mass cultures, may already contain various mutations in the heterozygous condition (H. A. and N. W. Timoféeff-Ressovsky, 1927; Tschetverikov, 1928). After being inbred in the course of the experiments they will show recessive "mutations," not freshly arisen but due to the segregation of some of the mutant genes already present in some concentration in the original stock.

The *second* and *third requirements* are: sufficiently large numbers of individuals and cultures in both the controls and the treated material, and genetic methods (types of crossings) suitable for the detection of newly arisen mutations. These two requirements are intimately connected with one another. It is self-evident that the numbers must be large enough to give sound results; but they must also correspond to the number of treated gametes. If five mice (*e.g.* males) are rayed and crossed to five untreated females, and say thirty F_1 individuals are raised, it will mean that only thirty treated gametes can be analysed in further generations. It must also be remembered that autosomal recessives will in most cases not show themselves before F_3 . Thus, sufficiently large numbers of F_1 individuals (corresponding to the number of treated gametes) must be bred and these F_1 individuals must be adequately analysed (at least until F_3) in order to discover whether they contain newly arisen mutations. The criteria of experiments for the induction of mutations fulfilling these requirements have been described in a special paper by P. Hertwig (1932 *b*).

The *fourth requirement* is the exact analysis of the variations arising, which can be of different types: (1) non-heritable modifications, (2) plasmatic enduring modifications (*Dauer-modifikationen*), (3) gene mutations, (4) chromosomal abnormalities. An exact analysis can only be performed if a genetically suitable organism has been chosen as the object of the experiments.

The *fifth requirement* is some knowledge concerning the manner in which the agent used can act on the germ cells of the treated object. Such agents as, for instance, X-rays or γ -rays always penetrate directly to the chromosomes of the treated germ cells. But in many other cases it must be demonstrated that the agent used can really reach the chromosomes of the germ cells of the object (*e.g.* in the cases of ultra-violet rays, visible light, or chemical treatments). It is certainly not impossible that some agents, although unable to penetrate directly to the germ cells, can nevertheless cause mutations indirectly by certain chemical changes induced primarily in some other parts or tissues of the treated organisms. But, in any case, this must so far as is possible be proved.

All the above requirements must be fulfilled in experiments designed to establish new viewpoints or to solve the principal questions in genetics. But the fulfilment of the first three of these requirements is absolutely necessary in any experiment dealing with the influence of any agent whatever on the heritable variation of organisms.

IV. RADIATION GENETICS.

In the problem of the experimental induction of heritable variations, radiation genetics—the production of mutations by short-wave radiations—occupies, both quantitatively and qualitatively, not only the first, but almost an exclusive place. Not only do most of the exact experiments on the induction of mutations lie at present within this field, but also the most interesting theoretical attempts to analyse the nature of the gene and of the process of mutation are connected with the use of short-wave radiations. Thus, radiation genetics will also occupy by far the most important place in the present review.

(1) *Historical attempts, and the first experiments of H. J. Muller.*

History. Soon after the discovery and elaboration of the physical properties of X-rays and radium, biologists and physicians emphasised the importance of these agents in attempts to affect the internal delicate structures of cells and tissues. Special histopathological work was done first by Bardeen (1906) and then by Regaud and Dubreuil (1908) and by O. Hertwig (1911, 1913) and his associates on animal germ cells and by Gager (1908) and Guilleminot (1908) on plants. Since then a great deal of work has been done in this direction showing that different structures in cells, including the chromosomes, can be affected by X-rays and radium. But, as was already mentioned, no strictly genetic tests were made in any of these experiments.

As early as 1920 some strictly genetic experiments with X-rays and radium were started. The most conclusive results were obtained by Nadson and Philippov, who succeeded in inducing new stable races of fungi (Nadson, 1920, 1925; Nadson and Philippov, 1925, 1926, 1928, 1931, 1932). Stein succeeded in inducing *Radio-morphose*, a cancer-like tissue abnormality, in *Antirrhinum majus* by radium treatment (Stein, 1922, 1926, 1927, 1929, 1930). This abnormality proved to be heritable

(Stein, 1932 b). Special tests on mice were performed by Bagg and Little and by Dobrovolskaia-Zavadskaia in order to induce mutations by X-rays (Little and Bagg, 1923; Bagg and Little, 1924; Dobrovolskaia-Zavadskaia, 1928). But the results obtained were inconclusive, although several mutations were probably induced by X-rays.

Approximately at the same time another series of experiments was started upon the influence of X-rays and radium on crossing-over and on non-disjunction of the X-chromosomes in *Drosophila melanogaster*. Mavor showed that X-rays influence the percentage of crossing-over in the X- and the II-chromosomes and raise the percentage of non-disjunction in this species (Mavor, 1921, 1922, 1923, 1924; Mavor and Svenson, 1924). He also showed that these effects are due to a direct influence exerted by the X-rays on the chromosomes of the germ cells (Mavor, 1929). Plough (1924) found that radium produces the same effects as X-rays on crossing-over and non-disjunction. Muller studied the differential effects of X-rays on crossing-over in different parts of the X-, II-, and III-chromosomes in *D. melanogaster* (Muller, 1925, 1926), and succeeded in producing genetically detectable chromosome breakages in the same species by X-rays (Muller and Dippel, 1926).

But all the above experiments, although showing that the germplasm can be affected by short-wave radiations, did not solve the problem of artificially inducing mutations, because they were either performed on unsuitable material and with inexact technique, or (as the *Drosophila* experiments on crossing-over) they dealt with other special problems. The question of the production of mutations by short-wave radiations was first definitely solved by Muller's X-ray experiments on *D. melanogaster* (Muller, 1927, 1928).

H. J. Muller's experiments. Muller's discovery of the pronounced effect of X-rays on the process of mutation was not a matter of chance, as has been the case in many other discoveries. His success was rather due to a very thorough and ingenious theoretical and technical preparation of the experiments. His experiments were the first which exactly fulfilled *all the requirements* enumerated above, showing at the same time that these requirements *must* be fulfilled in any exact experiment dealing with induction of mutations.

Since 1919, Muller has studied quantitatively the normal, spontaneous process of mutation in *D. melanogaster* (Muller and Altenburg, 1919; Muller, 1923, 1927 a, 1928 b). With the help of specially adapted, exact breeding methods he was able to show that it is possible to detect all sex-linked mutations arising in a certain number of gametes. (He found that the rate of mutations in the X-chromosome of *D. melanogaster* is measurable and equals about 0.1 per cent. By far the majority of the detectable mutations were found to be recessive lethals, producing no effect in the heterozygous condition, but killing the organism if homozygous.) Muller synthesised special cultures and described a method of crossing which allowed the easy and exact detection of all the sex-linked lethals arising in the sperm cells. Using exact methods of breeding and taking into consideration the whole experience and knowledge of *Drosophila* genetics accumulated since the beginning of the

genetic work with this species in Morgan's laboratory (Morgan, Bridges and Sturtevant, 1925), Muller could perform his X-ray experiments most critically.

His technique was as follows. Flies containing one or more mutant genes in their X-chromosome as markers were X-rayed in small gelatine capsules (50 kV., 5 mA., 1 mm. aluminium, dosage varied) and then crossed to untreated flies with a different constitution of the X-chromosomes. In the progeny of these crossings the treated (and marked) X-chromosomes could be followed and all mutations which arose in them during treatment could be detected.

Of special importance are two methods of crossing, which now are used in all

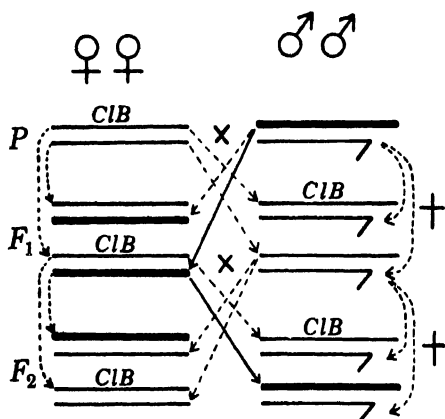


Fig. 1.

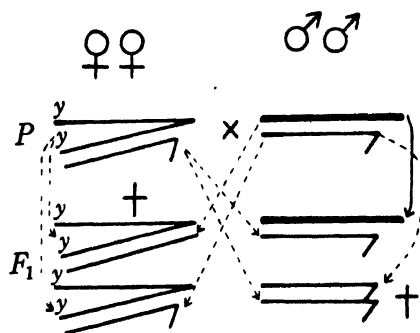


Fig. 2.

Fig. 1. Scheme of the *ClB* crossings in *D. melanogaster*; these crossings are especially suitable for the detection of induced sex-linked lethals. One of the X-chromosomes of the females contains an inhibitor of crossing-over (C), a recessive lethal killing the males (l) and the gene *Bar* as marker (B). *P* ♂♂ are rayed and in *F*₂ of *ClB* *F*₁ ♀♀ arising from sperms which contain a newly arisen lethal no males will appear at all. The rayed X-chromosomes are represented by darker lines.

Fig. 2. Scheme of the *attached-X* crossings in *D. melanogaster*; these crossings are especially suitable for the detection of induced sex-linked visible mutations. The two X-chromosomes of the females (containing the recessive gene yellow body colour as marker) are fused at their spindle-fibre ends; all surviving *F*₁ ♂♂ get their X-chromosome from the father. *P* ♂♂ are rayed and all visible sex-linked mutations induced in their sperm cells will show in the *F*₁ ♂♂. The rayed X-chromosomes are represented by darker lines.

exact mutation experiments with *D. melanogaster*: (1) the *ClB* method, and (2) the *attached-X* method. The scheme of the first of these is shown in Fig. 1. The *P*₁ ♀♀ contain in one of the X-chromosomes a dominant inhibitor of crossing-over (C), a recessive lethal (l) killing the males which contain this chromosome, and the dominant gene *Bar*-eye (B) as marker; such females give in their progeny a 2 : 1 sex ratio, since half of the males (*ClB*) die. If *P*₁ ♂♂ are treated and a large number of *F*₁ *ClB* ♀♀ are tested by further crossings, all mutations arising in the treated and tested X-chromosomes will show in *F*₂ ♂♂; if lethals arise in the treated X-chromosomes, the corresponding *F*₂ cultures will give no males at all (because one-half of the males will be killed by the *ClB* chromosome and the second half by the new lethal). The other method is shown in Fig. 2. The *attached-X* ♀♀ (*XXX*Y) have both

their *X*-chromosomes fused at the spindle fibre ends and possess an extra *Y*-chromosome; half of the eggs formed by these females contain the attached *X*-chromosomes (\widehat{XX}) and the other half the *Y*-chromosome. The eggs with attached *X*-chromosomes, if fertilised by *X*-containing sperm, give inviable \widehat{XXX} super-females (a few of them sometimes survive, but are sterile); and if fertilised by *Y*-containing sperm, give *attached-X* females (\widehat{XXY}). The eggs with a *Y*-chromosome give, when fertilised, either the inviable *YY* combination or regular *XY* males. All sons of the *attached-X* females thus get their *X*-chromosome from the father; if the P_1 ♂♂ are rayed all visible mutations produced by the treatment in the *X*-chromosome of their sperm cells will already be detectable in the F_1 males. The *attached-X* culture was first found and described by L. V. Morgan (1922) and is

Table I. Results of the "first X-ray experiments" of Muller with *D. melanogaster* (50 kV., 5 mA., 1 mm. aluminium, 16 cm. distance; $t_1 = 12$ min.). (From Muller, 1928 c.)

Series	No. of P_1 - F_2 cultures		No. of sex-linked mutations			
	Started	Hatched	Lethal	Semi-lethal	Weak	Vigour
Controls	1011	947	1	0	0	0
X-rays t_1 - t_4	1015	783	91	17	9	11

Table II. Results of Muller's CIB experiments. Males were X-rayed (dosages t_2 or t_4) and mated with CIB females. (From Muller, 1928 c.)

Series	No. of fertile P_3 - F_2 cultures	No. of sex-linked mutations		
		Lethal	Semi-lethal	Visible
Controls	198	0	0	0
X-rayed, dosage t_2	676	49	4	1 +
X-rayed, dosage t_4	772	89	12	3 +

best suitable for the detection of visible sex-linked mutations induced in treated sperm cells.

Table I shows the results of Muller's first X-ray experiment (1928 c). The number of mutations in the X-rayed chromosomes was found to be about 150 times higher than in the controls (128 : 783 and 1 : 947 respectively). Table II shows the results of the first CIB experiment: males were X-rayed and mated with CIB females, and the mutations induced in the treated *X*-chromosomes were detected in F_2 males. These experiments gave the same result as the first ones: a very pronounced acceleration of the process of mutation by X-rays. A third series of experiments was performed with the *attached-X* method: males were rayed and crossed to \widehat{XXY} females. As in the CIB experiments, two dosages, t_2 and t_4 , were used (t_4 being twice as high as t_2). In 1490 F_1 ♂♂ from fathers treated with t_2 ,

61 showed visible abnormalities, some of which were mutations, identical with previously known ones; 86 $F_1 \text{ ♂♂}$, out of 1150 from fathers treated with t_4 , showed visible abnormalities.

Further tests of the induced variations showed that many of them were allelomorphous to or identical with mutations already known from the spontaneous process of mutation in *D. melanogaster*. It was found that, besides gene mutations, chromosome abnormalities and rearrangements (breaks, deletions, inversions, translocations) are also produced by X-ray treatment.

Thus, the results of Muller's experiments showed (1) that X-rays induce mutations at a very high rate, (2) that different kinds of mutations can be induced, and (3) that in general the induced process of mutation is very similar to the spontaneous. The latter is shown by the fact that in both cases the same types of mutations appear, that lethals are much more frequent than visible mutations, and that most of the induced visibles are homologous with spontaneous mutations.

Many special questions concerning the nature of the process of mutation and of X-ray action were already raised and partially answered experimentally in these first experiments of Muller. They will be discussed in later chapters.

Confirmations of Muller's results. Soon after the first publication by Muller (1927) several papers appeared confirming and partially extending his findings to other tissues or species (Hanson, 1928; Hanson and Heys, 1928; Patterson, 1928; Serebrovsky and associates, 1928; N. T.-R.¹, 1928; Weinstein, 1928; Whiting, 1928). Hanson showed that substantially the same results can be obtained with radium treatment as with X-rays. Patterson and N. T.-R. induced somatic mutations by X-ray treatment of eggs and young larvae of *D. melanogaster*. Whiting induced mutations by X-rays in the parasitic wasp *Habrobracon juglandis*.

At approximately the same time as Muller's first publications appeared, the papers of Gager and Blakeslee and of Stadler describing their X-ray and radium experiments with *Datura* (Gager and Blakeslee, 1927; papers of Blakeslee and colleagues, 1928) and with barley and maize (Stadler, 1928) were published. These experiments were started and performed independently of Muller's work and reached substantially the same conclusions: short-wave radiations induce gene mutations as well as chromosome abnormalities in the progeny of treated plants and seeds.

(2) General validity of the effects of short-wave radiations on the process of mutation.

Soon after the appearance of Muller's first papers the radiation work was extended to several other species and to many special questions relating to the process of mutation.

It was shown that different short-wave radiations (γ -rays of radioactive substances, X-rays of different wave-lengths, ultra-violet rays), and also free electrons (β -rays of radium, cathode rays), if properly applied, will induce all known types of heritable variations (gene mutations and different types of chromosome abnor-

¹Throughout this article the initials N. T.-R. are printed for N. W. Timoféeff-Ressovsky.

malities). Mutations can be induced in different tissues: mature and immature sperm, mature and immature eggs, early developmental stages of the germ track, and different somatic tissues.)

(The most intensive and detailed work was done on *D. melanogaster*. But extensive work has also been done on other organisms (maize, barley, *Antirrhinum*, *Nicotiana*, *Habrobracon*), while with still other species the results obtained show that they will react genetically in substantially the same way. The following organisms have already been used in radiation genetic work: (a) Protista: *Chilodon uncinatus* (MacDougall); (b) plants: Mucoraceae, *Sporobolomyces*, *Nadsonia* (Nadson and Philippov), wheat (Stadler, Sapehin, Delaunay), oats (Stadler), barley (Stadler), rye (Levitsky), maize (Stadler), cotton (Horlacher, Goodspeed), vetch (Levitsky), *Crepis* (Levitsky, Navashin), *Hyacinthum* (de Mol), *Nicotiana* (Goodspeed), *Datura* (Blakeslee), tomatoes (Lindstrom), *Mirabilis* (Brittingham) and *Antirrhinum* (Stubbe); (c) animals: *Apotettix* (Nabours), *Habrobracon* (Whiting), *D. melanogaster* (Muller and many others), *D. funebris* (N. T.-R.), *D. virilis* (Demerec, Fujii), *D. pseudoobscura* (Sultz), and mice (Dobrovolskaia-Zavadskaia, Snell)¹.

The above-mentioned facts lead to the conclusion that short-wave radiations exert a very general effect on the germ plasm. We must expect that, if properly applied, short-wave radiations will produce genetic changes in any treated organism, and probably in any tissue capable of genetic reactions.

Treatment with short-wave radiations is an effective and sure method for accelerating the process of mutation. It has also the advantages that the dosages applied can be exactly measured and can be varied both qualitatively and quantitatively. In connection with these advantages many special problems arise within the field of radiation genetics. A part of these are purely genetic, in the sense that the treatment is merely a method for producing the variations which serve as material for genetic analysis. Other problems are connected with the analysis of the action of rays on mutability, and, thereby, of the nature of the process of mutation.

(3) Relation between the quantity of radiation and the mutation rate.

The first question arising in any experiment dealing with the effects of treatment is the relation between the applied dosage and the reaction obtained. The first CIB experiments of Muller (1928 c) showed that there is a direct proportionality between the dosage of X-rays applied and the percentage of mutations induced (Table II). In the following years several special experiments were performed to determine exactly the relation of the induced mutation rates to the dosages.

The first special tests on *D. melanogaster*, using the CIB method, were made by Hanson. He treated the males with 150 mg. radium for 9 hours and varied the thickness of the filter. His results showed that the rate of sex-linked lethals was directly and simply proportional to the ionisation rate of the dosages applied (Hanson and Heys, 1929).

¹ Recently Astaurov (1933) has published the first positive results of his extensive radiation-genetic experiments on inducing mutations in the silkworm *Bombyx mori*. And Pirocchi (1933) describes mutations induced by X-rays in *Macrosiphum rosae*.

Oliver (1930) performed a similar test using X-ray treatment. The quality of rays (50 kV. and 1 mm. aluminium) was kept constant; the dosage was varied by varying the time of exposure and was measured in r. units; dosages from 285 to 4560 r. were used. The results showed a direct, linear proportion to the dosage. Substantially the same results were obtained in experiments by Schechtman (1930) and by Efromison (1931), using dosages from 1125 to 9000 r., and by N. T.-R. (1934), with dosages from 1200 to 4800 r. (Fig. 3).

Thus, a number of independent experiments, performed at different laboratories and using dosages from 285 to 9000 r., have shown that in *Drosophila* there exists a direct linear proportionality between the dosage (ionisation rate) of radiation and the induced mutation rate. This regularity seems to hold also for different special types of mutations: unpublished data of N. T.-R. show that with the doubling of X-ray dosage the rate of induced sex-linked visible mutations is also doubled; and

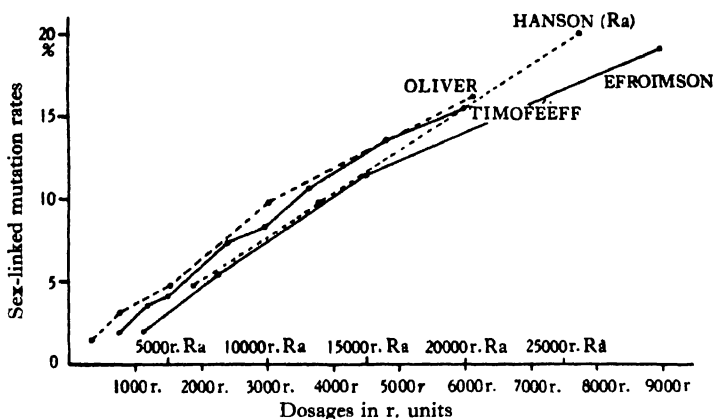


Fig. 3. The proportionality of the rates of sex-linked mutations in *D. melanogaster* to the dosages applied. X-ray experiments of N. T.-R. (1934), Schechtman (1930), and Oliver (1932), and radium experiments of Hanson and Heys (1932).

unpublished data of Muller suggest that the same is true in the case of induced breaks of the X-chromosome.

Stadler (1930, 1931), even before the above-mentioned *Drosophila* experiments were published, found that in barley and maize the rate of induced mutations also shows direct and probably linear proportionality to X-ray dosage.

Stubbe (1933), working on *Antirrhinum*, finds in his last publication that the rate of induced mutations rises until the X-ray dosage of 400 r. is reached, then it drops and begins to rise at a dosage of 3200 r. (the dosages used were: 100, 200, 400, 800, 1600, and 3200 r.). He gives a rather complicated theoretical explanation of this phenomenon. He assumes that low dosages of X-rays produce mutations only in certain labile genes; when higher dosages (over 400 r.) are reached, these labile genes begin to mutate to lethal allelomorphs, causing the death of mutated gametes and, correspondingly, the mutation rate drops; if still higher dosages are applied, other, stable, genes begin to mutate and the mutation rate begins to increase again.

But the experimental results of Stubbe are based on insufficient numbers of tested plants, so that even the largest differences between the rates of mutations within the range of higher dosages (400 and 1600 r.) are not statistically significant.) In the light of the exact results obtained in *Drosophila*, barley and maize, this type of relationship between dosage and mutation rate seems to be improbable; and it even disagrees with the results obtained by Stubbe (1932) in his earlier experiments on *Antirrhinum*¹.

On the basis of the present results we must admit that the induced mutation rate is directly proportional to the ionisation rate of the radiation dosage and that this proportionality is probably linear (not following an S-curve)². Thus, we do not expect to find a minimal active dosage of X-rays and radium, below which no mutations can be induced. And the second conclusion is that a rather simple relation must exist between the electron hits and the mutation reactions.

Table III. Sex-linked mutations in *D. melanogaster* and equivalent radium dosages applied in different concentrations. (From Hanson and Heys, 1932.)

Dosages			No. of F_2 cultures	No. of lethal mutations	Percent. of lethals
mg. Ra	Exposure in hours	Dosage in r. units			
300	0.5	6315	637	30	4.71
4	37.5	6316	636	30	4.72
2	75	6315	626	29	4.57
300	1	12630	626	61	9.75
4	75	12632	622	60	9.65
2	150	12627	619	56	9.53
4	150	25263	366	74	20.22

Another type of experiment performed on *Drosophila* gives further, indirect, evidence in favour of the above conclusions. It is known that in many physiological reactions (of more or less complex nature) to X-rays and radium the so-called "time factor" plays an important role: the effects are less pronounced if the same quantitative dosage is applied in diluted or spaced form. Such experiments, using fractioned and diluted dosages, have also been performed on induced mutation rates in *D. melanogaster*.

Patterson (1931), using the *CIB* method, applied an X-ray dosage of 1220 r.: (1) continuously (in 10 min.), and (2) divided into eight fractions (of 75 sec. each), spaced over different periods of time (intervals between the fractions being 24, 12, 8, 1 or 0.5 hours in different sets of experiments). The fractioning had no influence upon the induced mutation rate.

¹ Even if further tests should prove the reality of the phenomenon, other explanations must be taken into consideration. The physicist, Dr B. Rajewsky, proposed, for instance (in a discussion), as an explanation of Stubbe's results, the assumption that low dosages of X-rays produce some chemical changes in the tissues, which secondarily induce mutations; this chemical induction ceases when higher dosages are reached and causes the first unexpected peak on the proportionality curve.

² To avoid misunderstanding it must be stated that the word "linear" designates a simple relation between agent and reaction; the empirical curve will, certainly, show some "saturation effects" when high enough mutation rates are reached (because of the occasional coincidence of two or more induced mutations per gamete).

Hanson and Heys (1932) performed *CIB* experiments applying equivalent radium dosages of different concentrations. Table III shows that the dilution of the dosage had no effect on the percentages of mutations induced.

Table IV shows the results of the *CIB* experiments of N. T.-R. (unpublished),

Table IV. *Sex-linked mutations in D. melanogaster produced by equivalent concentrated, diluted and fractioned X-ray dosages.*

(Timoféeff-Ressovsky, unpublished data.)

Dosage and nature of treatment	No. of F_1 - F_2 cultures	No. of sex-linked lethals	Percent. of sex-linked lethals
Controls	1827	2	0.11
3600 r.; continuous in 15 min.	493	54	10.9
3600 r.; continuous in 6 hours	521	60	11.5
3600 r.; fractioned, 6 x 5 min., every 24 hours	423	47	11.1

where equivalent X-ray dosages were applied in concentrated, diluted and fractioned form. Neither the dilution nor fractioning of the dosage had any effect on the rate of induced mutations.

The above experiments, proving the absence of an effect of the "time factor" on the induced mutation rates, again show the simple proportionality of the percentage of mutations to the ionisation rate of the dosage applied.

(4) *Quality of radiation as related to the process of mutation.*

Limits of radiation frequencies effective in producing mutations. (From the shortest rays, the γ -rays of radium (Hanson and Heys, 1928, 1929 *a*; Stadler, 1928, 1930, 1931) to the softest X-rays (Efroimson, 1931; Schechtman, 1930; Stubbe, 1933), within the range of wave-length from 0.01 to 2.0 Å., all kinds of rays produce mutations in abundance.)

It is much more difficult, however, to test whether ultra-violet rays are effective in producing mutations. The experiments of Altenburg (1928, 1930) on ultra-violet treatment of *D. melanogaster* gave negative or inconclusive results as did Stubbe's (1932) ultra-violet experiments on *Antirrhinum majus*¹. In experiments of MacDougall (1929, 1931) on the infusorian *Chilodon uncinatus*, gene mutations and chromosome abnormalities were produced by ultra-violet rays. Results showing some positive effect of ultra-violet treatment on the mutability of *D. melanogaster*, although statistically insignificant, were obtained by Geigy (1931) and by Promptov (1932). The trouble is that in most cases, even in an object as small as *Drosophila*, the ultra-violet rays are absorbed in the surface tissues and do not penetrate to the gametes.

¹ In recent, still unpublished, work Stubbe has treated *Antirrhinum* pollen with ultra-violet and visible light of different wave-lengths (Noethling and Stubbe, 1934). He found a statistically significant increase of the rate of mutation following the treatment of pollen cells with ultra-violet rays of about 300 mm. wave-length. Treatment with visible light had no influence on the rate of mutation. These experiments show that ultra-violet rays are effective in inducing mutations, if suitable objects (allowing the rays to penetrate into the chromosomes) are used. I am very much obliged to Dr H. Stubbe for the permission to use his unpublished data. Recently Altenburg (*Science*, 78, 1933) also got positive results in *Drosophila*, in treating the "germ pole" of developing fertilized eggs with ultra-violet light.

Hanson (unpublished) and N. T.-R. (1931 *a*) independently found that the chitinous tergites and a tissue layer 0.5 mm. thick of *Drosophila* absorb almost all ultra-violet rays. Further treatments of Protozoa, pollen cells and perhaps of *Drosophila* eggs and young larvae, may yield definite results, which would be of great importance because ultra-violet rays of different wave-lengths have different specific photo-chemical actions. In applying various parts of the ultra-violet spectrum we can hope to exert specifically differentiated influences on the process of mutation.

Free high-speed electrons (β -rays of radioactive substances and cathode rays), if they penetrate into the gametes, probably produce mutations in the same way as do the X-rays. The effectiveness of β -rays was proved by Hanson in *D. melanogaster* (Hanson and Heys, 1928, 1929 *a*; Hanson and Winkleman, 1929), by Gager and Blakeslee (1927) in *Datura* and by Stadler (1928, 1930, 1931) in barley and maize. N. T.-R. found in a preliminary test that a substerile dosage of cathode rays slightly raises the percentage of sex-linked lethals in *D. melanogaster*. The low effectiveness is probably due, as also in the case of ultra-violet treatments, to the poor penetration of cathode rays, most of them being absorbed before reaching the chromosomes of the gametes.

Attempts to induce mutations in *D. melanogaster* by electricity (Horlacher, 1930; Schmitt and Oliver, 1933) and by supersonic vibrations (Hersh, Karrer and Loomis, 1930) gave negative results.

Thus, the above-mentioned facts show that all kinds of ionising radiations capable of penetrating into the gametes will produce mutations in abundance. The work with ultra-violet rays, being photo-chemically of special interest, is technically difficult because of the low penetration power and the pronounced physiological actions of these rays.

Relation between the quality of rays and the mutation rate. The discovery that rays of various wave-length produce mutations brings us to the next problem: the quantitative comparison of the action of qualitatively different rays. Such experiments have been performed within the range of different X-rays.

Schechtman (1930) and Efromson (1931), working on *D. melanogaster* with equivalent dosages of very soft (1.75 Å.) and hard (0.22 Å.) X-rays found that, if a correction for the lower penetration of the soft rays is made, equal dosages (in r. units) of soft and hard X-rays produce approximately equal percentages of sex-linked lethals.

Hanson, Heys and Stanton (1931) varied the voltage from 40 kV. to 99 kV. in their X-ray experiments on *D. melanogaster* and found that the rate of induced mutations remains proportional to the ionisation rate of the dosages applied, regardless of the wave-lengths of the rays.

Table V shows the results of *CIB* experiments on *D. melanogaster* by N. T.-R., using equivalent dosages (approx. 3600 r.) of soft (25 kV., 0.5 mm. aluminium) and hard (160 kV., 0.25 mm. copper + 3 mm. aluminium) X-rays. The percentage of induced sex-linked mutations was in both cases practically identical.

(Stubbe (1933), using equivalent dosages of very soft (8–10 kV.), soft (30–70 kV.) and hard (125–175 kV.) X-rays, found no statistically significant differences in the rates of induced mutations in *Antirrhinum majus*.)

In *D. melanogaster*, where very many visible mutations are induced by X-rays and radium, no qualitative differences in the mutabilities (differences in the kind of induced mutations) following treatment with different X-rays and radium can be detected.

All the above experiments show that within the range of X-rays the wave-length has no specific significance in the production of mutations. This is to be expected if the physical and photo-chemical properties of X-rays are taken into consideration. Of great importance would be the comparison of the effects on mutability of the different ultra-violet rays (having different photo-chemical actions) and of free electrons of different speeds (cathode rays at different voltages), if the technical

Table V. *Relation between quality of rays and the rate of sex-linked mutations in X-ray experiments with D. melanogaster. Soft and hard X-rays given in equal quantities (3600 r.), produce the same numbers of mutations, showing independence between mutation rate and quality of X-rays. (Timoféeff-Ressovsky, unpublished data.)*

Dosage	No. of cultures	No. of mutations	Percent. of mutations
Approx. 3750 r.; 25 kV., 0.5 mm. aluminium	486	63	12.9
Approx. 3750 r.; 160 kV., 0.25 mm. copper + 3 mm. aluminium	516	64	12.4
Controls	1827	2	0.11

difficulties caused by the low penetration power of these rays could be surmounted (using suitable objects, *e.g.* plant pollen or Protozoa, or suitable developmental stages of higher animals¹). Also an exact comparison of the mutation rates, induced by equivalent dosages of γ -rays and X-rays is still wanted².

(5) *Various conditions which might have an influence on the induced process of mutation.*

In the preceding chapters experiments were reviewed in which the quality or the quantity of the radiation applied was varied, all other conditions being kept constant. Now we will analyse various other conditions, which could have an influence upon the mutability induced by radiations.

Stability of different genes. The first question arising is whether or not different genes are equally susceptible to radiation. From our findings as to the spontaneous mutability of *D. melanogaster*, we know that different genes certainly have different mutation rates, and, consequently, different degrees of stability as regards those factors which produce the "spontaneous" mutations (Morgan, Bridges, Sturtevant, 1925; Muller, 1923). The rates of spontaneous mutations of different genes vary from 1 to more than 50 in the several millions of *D. melanogaster* flies analysed.

¹ See footnote on p. 422.

² Dr A. Pickhan, working at this laboratory and using exactly comparable and equivalent dosages of γ -rays of radium and of X-rays, found no difference in the mutation-inducing power of these rays (unpublished).

Similar conditions have been found in other well-analysed forms, as *Antirrhinum* (Baur, 1924) or maize (Stadler, 1930 b).

The X-ray work with *Drosophila*, *Antirrhinum* and maize shows that the frequency of induced changes is also different for different genes. In general, in *D. melanogaster* most of the frequent spontaneous mutations are also frequently induced by X-rays (Muller, 1928 d, 1930 a; N. T.-R., 1931 a). But probably exceptions will be found to this rule, as is already the case in maize (Stadler, 1930 b).

Different allelomorphs of the same gene may also show different degrees of stability in X-ray experiments. Within the white-eye series of allelomorphs in *D. melanogaster* the darker allelomorphs mutate more frequently under the influence of the same X-ray dosage than do the lighter allelomorphs (N. T.-R., 1932 c, 1933 b). Fig. 4 depicts the mutability of two different "normal" allelomorphs of this series, showing pronounced differences in the total frequency of mutations and in the relative frequencies of different mutational steps induced by X-ray treatment (N. T.-R., 1932 a, 1933 b).

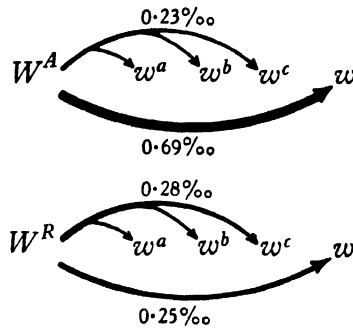


Fig. 4. Differences in the mutation rates from two different "normal" allelomorphs (W^A and W^R) of the white-eye series of *D. melanogaster*, to intermediate allelomorphs (w^a , w^b , w^c) and to white (w), induced by the same X-ray dosage (4800 r.).

Mutability in different species, races and individuals. The fact that different genes and even different allelomorphs of the same series have different stabilities makes it rather difficult to compare the mutabilities induced in different species, races or individuals by the same X-ray dosage. It is hard to say whether the differences obtained are due to some specific, racial or individual factors of a general kind, or simply to the fact that one of the groups contains a number of more or less stable genes or allelomorphs as compared with the other group.

The only clear and convincing results in this field were obtained by Stadler on X-ray induced mutation rates in related plant species showing polyploid series of chromosome numbers. He worked on four species of oats and on four species of wheat. *Avena brevis* and *A. strigosa* have a haploid number of chromosomes (7). The same number in *A. byzantina* and *A. sativa* is 21, showing that they have probably a triploid set of chromosomes. The haploid chromosome number is 7 in *Triticum monococcum*, 14 in *T. dicoccum* and *T. durum*, and 21 in *T. vulgare*, showing also a polyploid series. Mutations could be induced in a high rate in

Avena brevis, *A. strigosa*, and *Triticum monococcum*, the species with the simple set of chromosomes. In *T. dicoccum* and *T. durum*, the species with double sets of chromosomes, the induced rates of mutations were much lower, and in *Avena byzantina*, *A. sativa*, and *Triticum vulgare* (having 21 chromosomes) no mutations were induced at all (Stadler, 1929.) These results of Stadler are shown in Table VI and can be interpreted in the following way: in polyploid species most of the genes are present in double (or triple) number, so that most of the recessive mutations cannot manifest themselves even in homozygous condition, as they are covered by the normal allelomorphs present in the other homologous chromosomes.)

In experiments of H. A. and N. W. Timoféeff-Ressovsky more sex-linked mutations were induced in *D. melanogaster* than in *D. funebris* (by treatment with the same X-ray dosage). But at least a part of this difference is due to the more exact method of detection of sex-linked lethals in *D. melanogaster* (H. A. Timoféeff-Ressovsky, 1930 a, b; N. T.-R., 1931 a). In comparing sex-linked mutation rates (or in general, the mutation rates of certain single chromosomes) in different

Table VI. Relation between the induced mutation rates and chromosome numbers in related species showing polyploid series (oats and wheat). (From Stadler, 1929.)

Species	Haploid number of chromosome	No. of cultures	No. of mutations	Mutations per 1 r. unit $\times 10^{-8}$
<i>Avena brevis</i>	7	394	5	4.1
<i>A. strigosa</i>	7	1116	9	2.6
<i>A. byzantina</i>	21	337	0	0
<i>A. sativa</i>	21	413	0	0
<i>Triticum monococcum</i>	7	133	4	10.4
<i>T. dicoccum</i>	14	107	1	2.0
<i>T. durum</i>	14	444	6	1.9
<i>T. vulgare</i>	21	745	0	0

species the possible differences in relative "genetic" sizes (number of genes contained) of these chromosomes must also be taken into consideration.

No exactly analysed cases of differences in the induced mutation rates between races or individuals within a species are known which are not due to the presence of single frequently mutating genes. Serebrovsky found that in *D. melanogaster* the number of mutations obtained from different X-rayed individuals varies according to chance distribution, thus showing that there are probably no especially "mutable" individuals in this species (Serebrovsky *et al.*, 1928). The same was proved to be true by N. T.-R. (unpublished data).

From cases where different mutation rates are induced by the same dosage in different species or races, conclusions as to the different degrees of susceptibility of these groups to X-rays must be drawn very carefully. From Stadler's results with polyploid species we have already seen that the doubling of the set of chromosomes can mask the detection of mutations. In *Drosophila* we know many mutations suppressing other mutant characters ("specific suppressors," Bridges); and it is evident that races homozygous for suppressors of relatively frequently mutating

genes will not show these mutations, typical for other, related, races or species. Besides the *karyotypic masking*, due to the doubling of a part or of the whole set of chromosomes, and the *genotypic masking*, due to specific suppressors, we must reckon with the possibility of *phenotypic masking* of mutations in related species or races. The latter is due to the epistatic covering of certain mutant characters (making them undetectable) by some already present mutant characters. These theoretical considerations are mentioned in order to show at least a part of the difficulties which will be encountered in comparing the mutability of different species. Much further work must still be done in this direction.

Induced mutability in different sexes and tissues, and under different physiological conditions. In all tissues and cells so far tested, in which mutations can be detected, it has been found that radiation treatment induces mutations. Here we will mention some tests in which an exact comparison of the induced mutation rates in different tissues and under different conditions was carried out.

Muller (1929) found that in *D. melanogaster* the same dosage of X-rays induces

Table VII. Number of sex-linked lethals in sperm which was in different developmental stages at the time of the X-raying of males. The P_1 ♂♂ were X-rayed, mated with *CIB* ♀♀ and then every 5 days they were mated to new virgin *CIB* ♀♀. (From Timoféeff-Ressovsky, 1930 d.)

Age of sperm in days after X-raying	No. of fertile F_2 cultures	No. of sex-linked lethals	Percent. of lethals
1-5	417	29	6.9
5-10	491	41	8.3
10-15	481	35	7.3
15-20	478	19	4.0
20-25	411	13	3.1
25-30	389	7	1.8
Controls	984	0	0

more mutations in mature sperm than in the various developmental stages of the

The *Drosophila* males, after they have hatched from the pupa, already contain a certain number of mature sperm cells. When these are used up in the first copulations they are replaced by fresh ones, developing from the immature germ cells present in the gonads. Thus in an adult male, different developmental stages of the germ cells are rayed during the treatment. If treated males are mated every 4, 5 or 7 days to fresh virgin females, then in the first broods sperm will be used which has been X-rayed in the mature stage; and in the successive broods, sperm X-rayed in different immature developmental stages fertilises the eggs. Such experiments (using the *CIB* method) were independently performed by Harris, Hanson and Heys, and N. T.-R., and all gave the same results: the percentage of mutations decreases in subsequent broods, *i.e.* sperm X-rayed in mature stages contain more mutations than the sperm immature at the time of treatment (Hanson and Heys, 1929 b; Harris, 1929; N. T.-R., 1930 d). Table VII shows the results obtained by N. T.-R.

These results can be interpreted in two ways: (1) that the immature germ cells are much less susceptible to X-rays and the genes in them are much more stable, or (2) that at least a part of the difference in the induced mutation rates is due to germinal selection, since we were dealing with sex-linked lethals in male germ cells (having only one X-chromosome). It is known that genes are inactive in mature sperm of *Drosophila*, but they could influence the cell life and division rate in immature germ cells. N. T.-R. (1930 d, 1931 b) advocated the second interpretation, since he found, contrary to the results of Hanson, that the rate of non-lethal visible sex-linked mutations did not show any decrease in subsequent broods. The mortality in larval and pupal stages, caused by factors which probably do not directly influence the cell life, is equal after X-raying mature and immature sperm; but the mortality in the egg stage is much higher when mature sperm was rayed, probably because many of the induced factors influencing the early developmental stages are already underlying germinal selection if immature germ cells are rayed. A direct proof was furnished by Sidoroff (1931), who found that the percentage of autosomal lethals induced in the II-chromosome of *D. melanogaster* (which do not undergo germinal selection because the autosomes are present in diploid number in the immature germ cells) remains practically constant in all subsequent broods. But autosomal translocations are induced more frequently in mature sperm, as was shown by Schapiro (1931).

Another question to be solved was whether the process of the origin of mutations is bound up with some stages of chromosome division. The fact that mutations are frequently induced in dormant seeds (Stadler, 1930 a) and in mature sperm seems to disprove this assumption.) But it may be admitted that even in the mature sperm the chromosomes are not dormant and undergo, slowly, some preparations for further division. In this case, and if the process of the origin of mutations is confined to some of these stages of the chromosome cycle, we should expect to get different mutation rates by treatment of young and old mature sperm in *Drosophila*. Experiments of Harris (1929) and some other data show that the mature sperm already present in the young males is not being absorbed or ejaculated if they are not allowed to copulate. Thus, some of the males can be rayed just after they hatch from the pupae and others can be kept isolated for 20–25 days and then rayed. Such tests were made by N. T.-R. (1931 b) and gave no statistically significant difference between the mutation rates induced in young and old sperm by the same X-ray dosage (Table VIII). Thus, even if the chromosomes of mature sperm are not “dormant,” the origin of mutations is not restricted to some stage of chromosome division, since the frequency of this particular stage should be different in young and old sperm.

As was already stated, mutations can be induced in somatic tissues, giving rise to individuals showing mosaic distribution of the characters in question (Patterson, 1929 a, b; Stadler, 1930; N. T.-R., 1929 c). Patterson described the induction of somatic mutations (by X-raying eggs and larvae) in various tissues of *D. melanogaster*. But exact data on the rate of somatic mutations are present only for the sex-linked white-eye locus in *D. melanogaster* (Patterson, 1929 a). In Patterson's experiments

the rate of somatic white mutations was about 1 : 9000; the frequency of white mutations induced by the same dosage in mature sperm is at least twice as high. Thus, the rate of mutation of definite single genes can be different in different tissues.

That some physiological conditions can exert an influence upon the induced mutation rate in a definite tissue was shown by the fact that Stadler (1928, 1930 a), applying the same X-ray dosage to dormant and to germinating seeds of barley, got many more mutations from the treatment of germinating seeds.) Recent experiments of Hanson show that in *Drosophila* the rate of induced mutations may be influenced to some extent by starvation of the flies before or by anaesthesia during the X-ray or radium treatment (Hanson and Heys, 1933 a, 1933 b).

Relation between the induced mutability and various other agents combined with X-ray treatment. Agents other than short-wave rays can be applied in combination

Table VIII. Rate of sex-linked lethals, produced by X-rays in fresh and in old mature sperm cells of *D. melanogaster*. (From Timoféeff-Ressovsky, 1931 b.)

Types of cultures	No. of fertile F_1 cultures	No. of sex-linked lethals	Percent. lethals
Controls	984	0	0
Young males X-rayed and mated just after treatment	718	82	11.4
Old males, held 20-25 days without females, X-rayed and mated just after treatment	539	57	10.6

with X-ray treatment to test whether they influence the rate of mutation induced by X-rays. Two such agents have already been tested: (1) impregnation with salts of heavy elements, and (2) temperature.

Stadler (1928 b) showed that the impregnation of barley seeds with salts of heavy metals (barium nitrate, lead nitrate, and especially uranium nitrate) increased the effectiveness of X-ray irradiation, the rate of mutation being about 1 : 16 in impregnated and about 1 : 35 in non-impregnated seeds. The chemical treatment alone induces no mutations. The findings can be explained by the assumption that the impregnated seeds absorb more X-rays than do the chemically untreated ones.)

Stadler (1928 c, 1930 a) on barley and Muller (1930) and N. T.-R. (unpublished data) on *Drosophila* performed X-ray treatments at different temperatures (Stadler X-rayed barley seeds at temperatures of 10, 20, 30, 40 and 50° C., and found no effect of temperature (applied during the X-ray treatment) on the rate of induced mutations.) Muller X-rayed *Drosophila* males at 8 and 34° C.; N. T.-R. did the same at 16 and at 35° C. In both cases no effect of temperature could be detected. These results are of considerable importance, showing that the process of mutation induced by irradiation is probably based on reactions of the monomolecular type, which do not follow the Van't Hoff rule.

Further work in this direction, using other accompanying agents and, if possible, mutation rates of single genes, would be of great interest.

Direct or indirect action of short-wave rays on mutability. The simple proportionality of the induced mutation rates to dosages and the results of the experiments reviewed in the preceding paragraph suggest that the action of short-wave rays on the genes is rather a direct one, understanding "direct" as the immediate local action of the primary quanta or secondary released electrons. But *a priori* it does not seem improbable that at least a part of the genetic effects of X-rays are due to some more or less stable chemical changes primarily induced in the irradiated chromosome material or even in the cytoplasm. In this case a delayed "after-

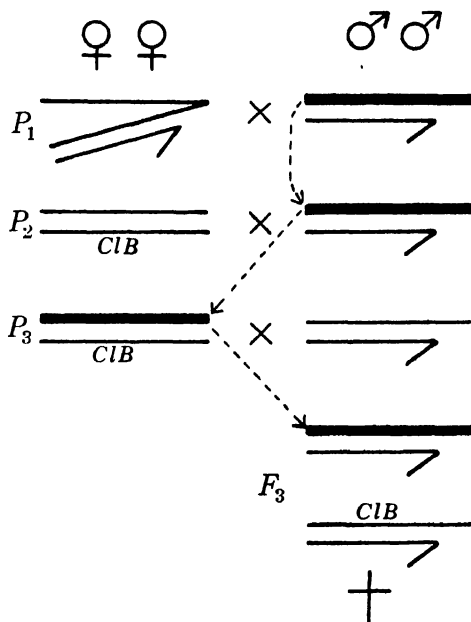


Fig. 5. Scheme of crossings made to test whether there is some "after-effect" of X-raying upon mutability in the next generations. $P \delta\delta$ are rayed and crossed to *attached-X* ♀♀ ; all $F_1 \delta\delta$ surviving and showing no new mutations contain an X-rayed X -chromosome in which no mutations arose during the treatment. These males are crossed to *CIB* ♀♀ and in F_2 all mutations arising in the previously treated X -chromosome can be detected. The treated chromosomes are represented by darker lines.

effect" would be detectable, increasing the rate of mutation in previously rayed chromosomes and in untreated chromosomes crossed into an X-rayed cytoplasm.

These latter assumptions can be tested experimentally in *D. melanogaster*. Fig. 5 shows the method of crossing suitable for the detection of an eventual "after-effect" of X-ray treatment on the rate of mutation. Normal $F_1 \delta\delta$ from crosses of X-rayed $\delta\delta$ to *attached X* ♀♀ contain an X-rayed X -chromosome in which no mutations arose immediately during the treatment; the males are then crossed to *CIB* ♀♀ and in F_2 from these crossings (*i.e.* in F_2 from the beginning of the experiment) the rate of mutation in the previously treated X -chromosomes can be determined. Such, or similar, experiments were independently performed by

Muller (1928 *c*, 1930 *a*), N. T.-R. (1930 *c*, 1931 *b*), and Grüneberg (1931), and all gave the same results: no "after-effect" of X-ray treatment on the rate of mutation could be detected.

Fig. 6 shows the method of crossing used by N. T.-R. (1931 *a*, *b*) in experiments destined to test if there is any influence of X-rayed cytoplasm on the rate of mutation in untreated X-chromosomes. Untreated males are crossed to X-rayed *attached-X* females; the F_1 ♂♂ from these crossings contain an untreated X-chromosome in rayed cytoplasm; the rate of mutation in their X-chromosomes is tested by further *CIB* crossings. These experiments showed that X-rayed cytoplasm has no effect at all upon the rate of mutation in untreated chromosomes.

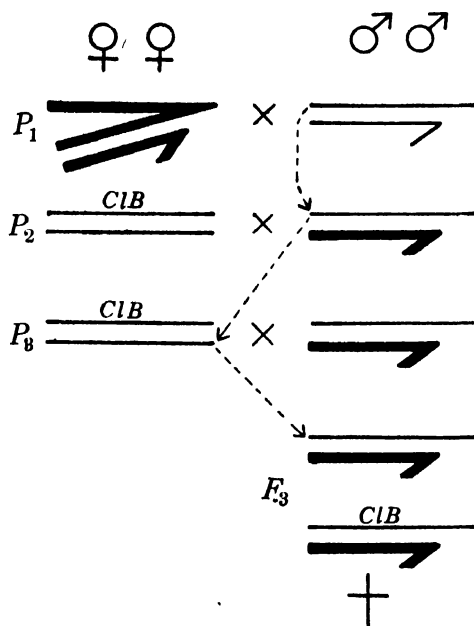


Fig. 6. Scheme of crossings made to test whether the X-ricing of the egg plasm has an influence on the origin of mutations in untreated chromosomes. *Attached-X* ♀♀ are rayed and crossed to untreated ♂♂; the F_1 ♂♂ thus contain untreated X-chromosomes in X-rayed plasm; they are crossed to *CIB* ♀♀ and in F_3 the percentage of mutations arising in the untreated X-chromosomes within the treated plasm can be determined. The treated chromosomes are represented by darker lines.

The results of the experiments of N. T.-R. (1930 *c*, 1931 *a*, *b*) on the "after-effect" of X-rays and on the influence of X-rayed cytoplasm upon the rate of mutation in untreated chromosomes are shown in Table IX.

Thus, all the above experiments, as well as the indirect evidence mentioned at the beginning of this section, tend to deny the existence of an "indirect" effect of X-ray treatment upon mutability. On the other hand, there are some facts which could be explained by the assumption of an "after-effect." Muller (1928 *d*, 1930 *a*) found that X-ricing of mature *Drosophila* sperm induces a certain amount of "fractional mutations," *i.e.* half-to-half mosaics, showing the mutant character

only in one-half of their body. This can be explained either by an "after-effect" of the treatment (assuming that the delayed mutation occurs just after fertilisation, in the first cleavage stages), or by the assumption that a part of the mature sperm contains chromosomes that are already split, and that in this case the mutation arises only in one-half of a split chromosome. Muller himself tends to admit the latter explanation, since some unpublished cytological observations of Shiwago show that double strand chromosomes (already split for further divisions) are likely to occur even in the resting stages of the cells.

Table IX. *The frequency of sex-linked mutations in D. melanogaster: (1) in untreated controls; (2) in cultures, containing a previously X-rayed X-chromosome, which was free of mutations just after treatment; (3) in cultures, containing not-treated X-chromosomes in X-rayed egg plasma; and (4) in cultures with directly treated X-chromosomes. (From Timoféeff-Ressovsky, 1931 b.)*

Types of cultures	No. of fertile cultures	No. of sex-linked lethals	No. of sex-linked visible mutations
Untreated controls	793	1	—
Cultures with previously X-rayed X-chromosome	756	2	—
Cultures with untreated X-chromosomes in X-rayed egg plasma	581	—	—
Cultures with directly X-rayed X-chromosomes	844	89	8

(6) *Types of induced mutations and comparison of induced and spontaneous processes of mutation.*

Classification of heritable variations. All the different types of heritable variations must be divided into two groups: plasmatical changes (variations of the "plasmotype") and genotypical changes (variations of the "genotype"). We naturally exclude all so-called "combinations," due to hybridisation and not accompanied by real changes in the original germ plasm.

Our knowledge of *plasmatical changes* is still very scanty. But they probably involve the following three types: (1) changes in some structural elements of the cytoplasm (*e.g.* plastids in plant cells), (2) adaptation of the plasmotype to changed genotypical constitution (*e.g.* in some species hybrids in plants), (3) enduring modifications or *Dauermodifikationen* (*i.e.* induced, slowly reverting, changes in the plasmatic constitution).

The *genotypical changes*, or mutations, involving qualitative or quantitative changes in the set of genes (localised in the chromosomes), can be classified according to the unit of change: it can involve either a single gene, or (without changing the genes) a chromosome, or (without changing the single chromosomes) the set of chromosomes (the "karyotype"). Thus we can distinguish three types of mutations: (1) gene mutations (changes in the single genes, leading to the formation of new allelomorphs), (2) chromosome mutations (intrachromosomal rearrangements and

quantitative changes, the genes remaining unchanged), (3) karyotype mutations (changes in number of chromosomes, genes and chromosomes remaining unchanged). Gene mutations are changes in the gene structure leading to the origin of new allelomorphs of the genes in question. Mutant allelomorphs may behave as dominants, intermediates, or recessives; they can affect any organ or characteristic of the organism; they can exert a lethal or sublethal effect, lower the viability of the organism, leave it unaffected, or (in rare cases) even raise the viability of the mutant type; single mutant allelomorphs can produce visible effects in one or few characteristics of the organism, or affect several different characters ("pleiotropic genes"). The phenotypic manifestation of the mutant allelomorphs may be full and constant, or it can be variable and even dependent upon other factors (low penetrance, variable expressivity and specificity of the genes, N. T.-R., 1931 c). Chromosome mutations consist in breaks, deletions or section inversions within single chromosomes and in translocations of pieces within the same or to another chromosome. Various chromosome mutations are shown in Fig. 7. Karyotype mutations consist in changes of chromosome number, the structure of chromosomes being unchanged; addition of one or more whole sets of chromosomes results in polyploidy, and the addition or subtraction of one or several single chromosomes leads to trisomics or heteroploidy. Karyotype mutations are shown in Fig. 8.

Thus, the following classification of heritable variations may be given:

A. Plasmatical changes.

- (1) Changes in structural elements of the cytoplasm (Correns, 1909).
- (2) Adaptation of the cytoplasm to changed genotype (Michaelis, 1933).
- (3) Enduring modifications (*Dauermodifikation*, Jollos, 1913).

B. Genotypical changes.

- (1) Gene mutations (mutation *sensu stricto*).
- (2) Chromosome mutations.
 - (a) Breaks and fragmentations.
 - (b) Deficiencies and deletions.
 - (c) Inversions.
 - (d) Simple translocations and duplications (intra- and interchromosomal).
 - (e) Mutual translocations.
- (3) Karyotype mutations.
 - (a) Trisomics.
 - (b) Heteroploids.
 - (c) Polyploids.

Types of induced heritable changes. All types of heritable changes mentioned in the above classification were already known from the spontaneous process of heritable variability in *Drosophila* and in some plants. All these types have appeared in the progeny of X-ray or radium treated plants and animals; but, and this is a

very important fact, no new types, unknown from the spontaneous process of mutability, have ever been found in radiation genetical work.

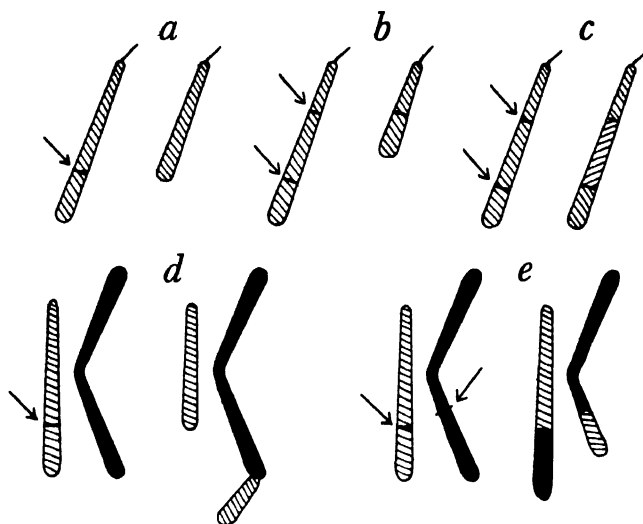


Fig. 7. Different types of chromosome mutations (chromosome breakage): *a*, simple break, followed by the loss of a part of the chromosome; *b*, deletion, following a double break; *c*, inversion of a section of the chromosome; *d*, simple translocation of a piece of one chromosome to another; *e*, mutual translocation. At the left is shown the normal and at the right the resulting mutant condition. Arrows indicate the points of breakage.

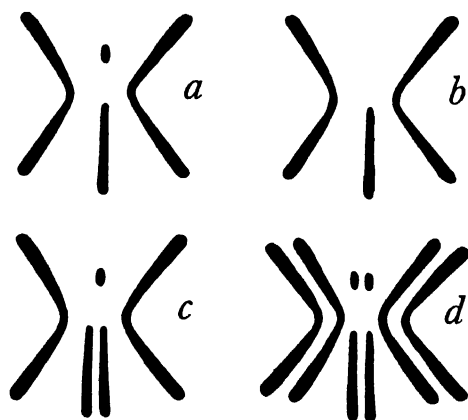


Fig. 8. Different types of mutations of the karyotype (changes of the chromosome number): *a*, a normal haploid (n) set of chromosomes; *b-c*, heteroploidy ($n-1$ or $n+1$, in general form, $n-m$ or $n+m$); *d*, polyploidy ($2 \times n$, in general form, $m \times n$).

(1) *Gene mutations*. In the X-ray and radium work, different kinds of gene mutations were induced in *Drosophila* and other tested animals and plants. In a number of species most of the induced mutations are recessive; the same is the case in the spontaneous process of mutation in these species. But dominants are

also induced at approximately the same rate (in relation to recessives) as they occur spontaneously. The induced mutations, as well as the spontaneous, can affect all parts and characteristics of the organism (Morgan, Bridges and Sturtevant, 1925; Muller, 1928 *d*, 1930 *b*). Besides morphological characters they can also affect different physiological characteristics of the organism, and its viability (N. T.-R., 1933 *d*). Gene mutations can be induced in somatic cells and produce mosaic patterns (Patterson, 1929 *a, b*; N. T.-R., 1929 *c*). Mutations can be induced not only from the "normal" allelomorphs of the wild type to "mutant" allelomorphs, but also from recessive mutants back to or towards the original normal

Table X. *Reversions of recessive gene mutations in the X- and III-chromosomes of D. melanogaster produced by X-rays (dosages 3600 and 4800 r.). (From Timoféeff-Ressovsky, 1933 e.)*

X-rayed allelomorphs and their loci	Number of analysed X-rayed chromosomes containing the loci	Type and number of reversions
X-chromosome, o y	11,781	—
„ o + sc ¹	17,676	3 sc ¹ → Sc
„ 2 w	29,233	1 w → w ^o
„ 2 w	29,233	1 w → w ^b
„ 2 w ^o	23,472	1 w ^o → w ^b
„ 2 w ^e	23,472	1 w ^e → W
„ 7 ec	17,676	—
„ 16 cv	6,354	1 cv → Cv
„ 25 ct ⁸	12,914	—
„ 40 v	19,268	1 v → V
„ 51 g ²	12,914	—
„ 62 f	24,695	5 f → F
III-chromosome, o ru	16,936	—
„ 26 h	16,936	1 h → H
„ 42 th	5,681	—
„ 44 st	16,936	—
„ 48 p ^p	11,255	2 p ^p → P
„ 50 cu	5,681	—
„ 58 ss	11,255	—
„ 62 sr	5,681	—
„ 71 e ^o	16,936	1 e ^o → E
„ 101 ca	5,681	—
Total	288,961	18
Controls	139,234	0

allelomorphs (Patterson and Muller, 1930; N. T.-R., 1925, 1928 *a*, 1929 *a*, 1930 *a, b*, 1932 *c*, 1933 *a, b, c*). Table X shows the results of X-ray experiments on induction of such reverse gene mutations. The occurrence of reverse mutations is of great importance, showing that the process of mutation does not consist merely in a destruction of the normal wild-type allelomorphs. The different kinds of gene mutations were induced not only in *D. melanogaster*, but also in all other organisms which were studied extensively: *D. funebris* (H. A. Timoféeff-Ressovsky, 1930), *D. pseudo-obscura* (Schultz, 1933), *Habrobracon* (Whiting, 1929), maize, barley (Stadler, 1931), *Antirrhinum* (Stubbe, 1932, 1933).

(2) *Chromosome mutations.* In *D. melanogaster* all types of chromosome mutations (breaks, deletions, inversions, translocations) have been induced by X-ray

treatment. Heavy dosages produce them in such abundance that even certain types needed for some special cytogenetical work can be induced by will if large enough numbers of flies are treated. This opens a wide field for cytogenetic research (Dobzhansky, 1929 *b*, 1930 *b*, 1931, 1932; Muller and Altenburg, 1930; Muller and Painter, 1929, 1932; Painter, 1931; Painter and Muller, 1929, 1932). Fig. 9 shows an X-ray induced translocation from the II to the III chromosome in *D. melanogaster*, which was tested both genetically and cytologically. Chromosome mutations can also be induced in somatic cells (Patterson, 1929 *b*, 1930 *a*). Chromosome mutations have also frequently been induced in a number of plant species:

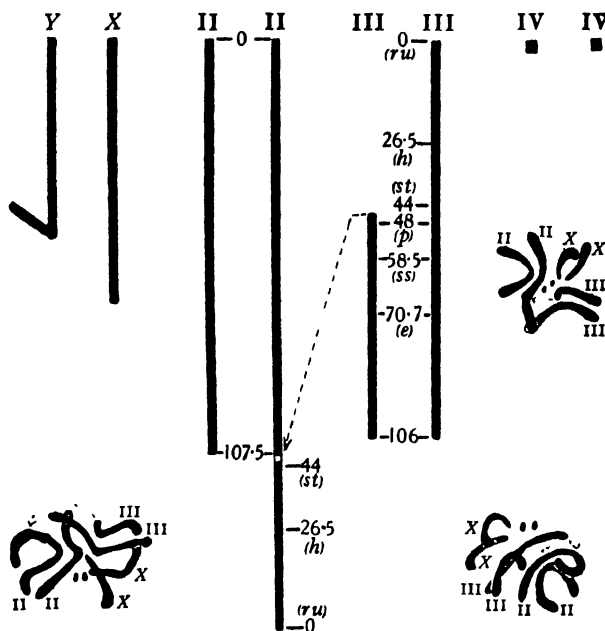


Fig. 9. An X-ray induced translocation of a large piece of the left arm of the III-chromosome to the right end of the II-chromosome in *D. melanogaster*, proved genetically and cytologically. (From Painter and Muller, 1929.)

Datura (Blakeslee, Avery, Bergner, Buchholz, Cartledge, Satina, 1928-9), *Nicotiana* (Goodspeed, 1930, 1931, 1932), maize (Stadler, 1931; McClintock, 1931), *Triticum* (Delaunay, 1930), *Secale*, *Vicia*, *Crepis* (Levitsky and Araratian, 1931; Navashin 1931).

(3) *Karyotype mutations*. Non-disjunction of the sex-chromosomes, leading to heteroploidy, was the first heritable change produced by X-rays in *D. melanogaster* (Mavor, 1921, 1922, 1924). Since then heteroploids and polyploids were frequently induced, especially in some plant species (Blakeslee, *et al.* 1928-30; Goodspeed, 1931; Levitsky and Araratian, 1931).

(4) *Plasmatic changes*. To this group perhaps belong some of the so-called "types," described by Stubbe (1932) in his X-ray work on *Antirrhinum*. These

abnormalities show inheritance only through the female, suggesting that they are caused by some factors localised in the cytoplasm. In *D. melanogaster* Woskresensky (1929) induced, by X-ray treatment, an acceleration of the time of development, which persisted for several generations and gradually disappeared. He designed this case as *Dauermodifikation*. The present author found in his X-ray experiments several variations in *D. melanogaster* and *D. funebris*, which were transmitted only by the females and gradually disappeared within three or four generations (N. T.-R., 1932 *b*). These cases do not fit any chromosomal or genotypic explanation and can best be explained as being due to plasmatic enduring modifications. But, as was already mentioned in the preceding section, our knowledge of the role of the cytoplasm in inheritance is still very meagre, and much further exact work must be done in this direction.

Comparison of the induced and spontaneous processes of mutation. It has already been stated above that no new types of "X-ray or radium mutations" were observed in any of the treated species, and that there is a far-going general parallelism between the induced and spontaneous processes of mutation.

A more or less detailed comparison can only be made in *D. melanogaster*, a species in which we already know several hundred different spontaneous and induced mutations. This comparison shows that most of the induced gene mutations are identical with, or allelomorphic to, already known spontaneous mutations. Some of the induced gene mutations are quite new; but every year new spontaneous mutations are also found. Both in the spontaneous and induced processes of mutation we observe about the same proportions of recessives and dominants, and of visibles and lethals, the latter being more than ten times as frequent as the non-lethals. Most of the recurrent spontaneous mutations have also been observed more than once in both X-ray and radium experiments; and such mutations as, *e.g.* *Bar*, which arose (spontaneously) only once in a very large number of flies, have not yet been induced by X-rays or radium. This means that in general (although many exceptions from this rule will probably be found) the same genes behave as stable or less stable ones in both the spontaneous and induced processes of mutation (see Table 33 in N. T.-R., 1931 *a*). As to the chromosome mutations, it has already been stated that all of these, induced in abundance in X-ray or radium experiments, belong to one of the types already known from spontaneous mutation. Here, however, there seems to be a difference: the relative frequency of chromosome mutations (as compared with gene mutations) is probably higher in the induced process.

Another *Drosophila* species, *D. funebris*, differs in the general type of its spontaneous mutation from *D. melanogaster* (it has more semi-dominant mutations, relatively more mutations with variable phenotypic manifestation, and some of the most common mutations of *D. melanogaster*, such as *white* or *yellow*, have never appeared in *D. funebris*), but the same general differences have been found in its X-ray induced mutability (H. A. Timoféeff-Ressovsky, 1930 *a*, 1930 *b*).

In most of the other species used in X-ray work the comparison has not yet been carried through in detail. But even if some specific differences are found, the

general similarity of the induced and spontaneous process of mutation is large enough to allow far-going deductions from the analytical radiation genetic work to the spontaneous mutability in different species.

(7) *The nature of the effect of rays on the process of mutation.*

From the results of the radiation genetic experiments reviewed above, some conclusions can be drawn upon the kind of action exerted by the short-wave rays on the process of mutation. The most important of these conclusions are the following.

(1) The action of the short-wave radiations upon the germ plasm is of a general kind. All hitherto known types of genetic changes can be produced by ionising radiation if the latter reaches the cells in question without killing the treated organism.

(2) Within the range of X-rays and radium-rays the genetic action of radiation seems to be non-specific: if equivalent dosages are used it is independent of the wave-length applied. Within the range, at least, of (soft and hard) X-rays the action is also quantitatively independent of wave-length, being simply proportional to the amount (the ionisation rate) of the dosage. A more or less specific action is only to be expected within the ultra-violet rays, since different parts of their spectrum have different photo-chemical actions. The shortest γ -rays can perhaps produce an effect somewhat different from that of X-rays, because the basic physical action on atoms of the former is probably somewhat different from that of the latter (ejection not only of electrons but also of positrons)¹.

(3) The action of short-wave radiation upon genes and chromosomes is a direct and simple one. This follows from the absence of a genetic "after-effect" of X-ray treatment, independence of the X-ray induced mutation rate from temperature (applied during X-ray treatment), absence of an influence of the "time factor" (dilution or fractioning of the dosage) upon the rate of induced mutations and from the simple direct proportionality of the induced mutation rates to the dosages. If mutations were produced, not directly by the radiation quanta or the released electrons, but indirectly by some physiological or chemical reactions primarily induced by radiation, then we should expect to find some of the above-stated complications in the relation between radiation treatment and induced mutation rate.

(4) Radiation is capable of producing mutations in different tissues, under different physiological conditions, and in the presence of different accompanying factors. But at least some of these secondary conditions and factors (*e.g.* rate of metabolism, impregnation with salts of heavy metals, etc.) can influence the rate of mutations induced by radiation treatment. This shows that the process of mutation is dependent not only upon the physico-chemical structure of the mutating units (genes or chromosomes), but also upon the nature of the chemical environment of these units.

(5) The different types of induced gene mutations show that the genetic action of short-wave rays is not merely destructive, but rather reconstructive, since

¹ See footnote 2 on p. 424.

"direct" and "reverse" mutations (*i.e.* mutations of the same gene in opposite directions) can be induced by irradiation. The absolute frequency of mutations is dependent upon the dosages, but the relative frequencies of different gene changes, and the direction of mutations, is determined by the structure of the genes in question.

(6) From the results of different radiation genetic experiments the following statements can be made concerning the nature of gene mutations and the structure of the gene. (a) The fact that reverse gene mutations (Table X) can be induced by irradiation shows that in general mutations are not merely losses of previously present genes. In several cases (Patterson and Muller, 1930; N. T.-R., 1930 *b*, 1932 *c*) "direct" and "reverse" mutations were induced by X-rays directly one from another in *D. melanogaster*. The following cases are examples. From a normal allelomorph of the white series eosin was induced, and the latter, under further treatment, produced a reversion back to normal. A spontaneously arisen eosin gave, under treatment, a reversion to normal, and this normal mutated under further treatment back to eosin. Mutations were produced by X-rays from normal to forked and from this forked back to normal, and from forked to normal and from this normal back to forked. Mutations were produced from pink to normal and from this normal back to pink. From these cases it becomes evident that the action of X-rays cannot be of a purely destructive kind and that the gene mutations, at least in these and in similar cases, cannot be simple losses of the previously present gene, or even of a specific part of the gene substance, because as Muller has expressed it, it is highly improbable that "if with one blow we punch the gene out, with the next we would punch it in again." The most plausible assumption would be, then, that gene mutations are reconstructions of the gene, *i.e.* some physico-chemical changes of its structure (N. T.-R., 1932 *c*). (b) The absolute frequency of gene mutations is determined by the dosage. But different genes, and even different allelomorphs of the same gene, give distinctly different relative mutation rates, thus showing that the structure of the gene is the main cause of its relative stability. In no case was it possible to find a dependence of the kind of induced mutations on the kind or the amount of radiation applied; this shows that the structure of the gene must also be responsible for the direction of its mutability. The X-ray induced mutability at the locus of white in *D. melanogaster* shows (Fig. 12) some characters of "determinate variation," the different mutational steps being not unordered but occurring with different specific frequencies (N. T.-R., 1930 *b*, 1932 *c*, 1933 *b*, *c*). This must also be determined by some specific characteristics of the gene structure. Thus we come to the conclusion that the structure of the genes of a given group of organisms determines to some extent the evolutionary potencies and the direction of evolution of this group. (c) Concerning the physico-chemical nature of the genes, two views can be confronted. The genes are either fixed quantities of specialised matter (consisting of several or even many equal physico-chemical units), or they are physico-chemical units (molecules, micellae, or colloid particles of specific structure). The former view is expressed and elaborated in Goldschmidt's quantitative theory of gene action and gene mutation (1928). This view, and Goldschmidt's theory in its relation to gene structure, is merely a specification of Bateson's "presence or

absence" hypothesis, and its difficulties in explaining many facts in our present knowledge about allelomorphic differences and mutation in different directions are just the same as those of the classical form of the "presence or absence" theory. The facts of reverse mutation, determinate mutability of some of the genes (N. T.-R., 1932 *c*, 1933 *b*, *c*), frequently mutating genes (Demerec, 1928, 1929), and of "non-serial" allelomorphic series (Dubinin, 1929, 1932; N. T.-R., 1932 *c*, 1933 *c*), are much easier to fit into the second view, *i.e.* that genes are physico-chemical units and gene mutations are changes in the structure (and, in consequence in the properties) of these units (Patterson and Muller, 1930; N. T.-R., 1930 *b*, 1932 *c*, 1933 *b*, *c*). This view may serve as a working hypothesis; our present empirical knowledge is far too insufficient to build up more detailed theories of the structure of the gene. But radiation genetics gives us new methods for attacking the gene problem.

(8) *Applications and conclusions.*

Applications of radiation genetic methods. Besides their own lines of development (*i.e.* the analysis of the action of short-wave rays upon the germplasm), some of the methods of radiation genetics can already be applied to the study of various general genetic questions. In some of these questions really exact work can only be done through the experimental induction of mutations by X-rays or radium. The methods of radiation genetics have already been applied with success in the following cases.

(1) *Induction of chromosome mutations.* One of the fields of application of X-ray induced chromosome mutations is cytogenetics and especially the elaboration of "cytological chromosome maps." An exact comparison of "genetical" and "cytological" map distances was first made by Muller and Painter (1929) and by Dobzhansky (1929). Now, through special investigations of Dobzhansky (1929 *b*, 1930 *a*, *b*, 1931, 1932) and of Muller and Painter (Muller and Painter, 1932; Painter, 1931; Painter and Muller, 1932), we are already in possession of preliminary "cytological maps" of the three long chromosomes of *D. melanogaster*. A comparison of the genetic and cytological maps of the *X*-, *II*- and *III*-chromosomes is shown in Fig. 10. Induced inversions, deletions, fragmentations and translocations of chromosomes are also used in studies on crossing-over and chromosome conjugation and disjunction, both in *Drosophila* and in maize.

Another new and important field of research connected with X-ray induced chromosome mutations is the study of the action of varying amounts of single individual genes. Although some work in this direction had already begun before the discovery of the genetic effects of X-rays (Bridges, Mohr, Stern), an effective and general attack on this problem is connected with experimental induction of chromosome mutations *en masse*. The first experiments dealing with this question were made by Muller (1932 *b*), who studied the effects of different individual genes in hyperploid combinations, using small fragments of chromosomes (containing the gene in question) induced by X-rays. Muller classifies mutations on their counter-action on the original allelomorph from which they arose. He distinguishes the following chief types of mutations: (*a*) hypomorphs, (*b*) hypermorphs, (*c*) antimorphs, (*d*) neomorphs, and (*e*) amorphs. The hypomorphs are mutations

producing the same, but less pronounced, effect as those allelomorphs from which they arose; if present in supernumerary condition, they approach the effect produced by the original allelomorphs from which they arose. Hypermorphs produce a stronger, but similar, effect as the original allelomorphs. Antimorphs are those mutant allelomorphs which produce an effect opposite to that of the original allelomorph: the phenotypic end-effect of different antimorphic combinations is the result of their antagonistic actions. Neomorphs produce an effect which is "new" for this gene: the original allelomorph is an "amorph" in respect to the

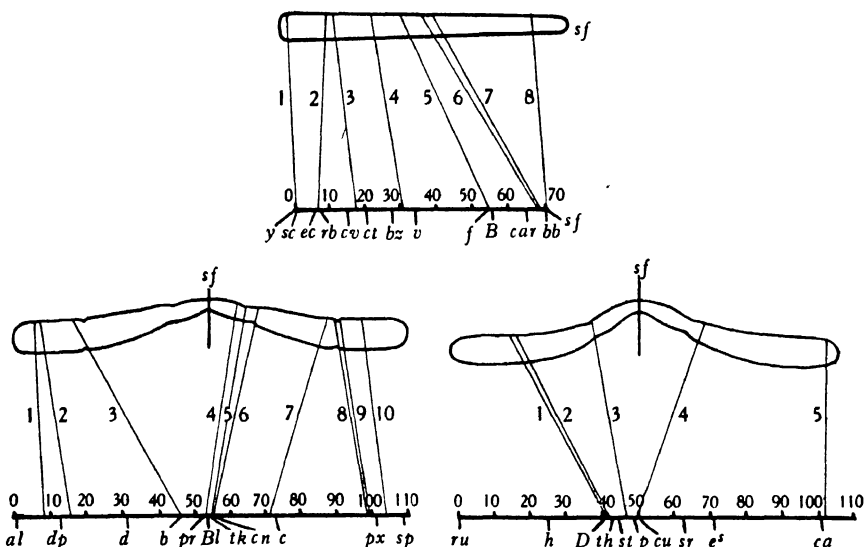


Fig. 10. A comparison of the genetic (crossing-over data) and cytological (cytological analysis of translocations from and to genetically known loci) maps of the X-chromosome (Muller and Painter, 1932), II-chromosome (Dobzhansky, 1930) and III-chromosome (Dobzhansky, 1929) of *D. melanogaster*. The lines 1, 2, 3, etc., connect the points of breaks on the genetic map and on the actual chromosome, showing, in both cases, identity of gene sequence but different relative distances between the genes.

character or characters produced by the neomorph and does not affect its development at all. Muller's classification of mutations, and further work in this direction (made possible by the application of radiation genetics), will bring us a step forward in our knowledge of the structure, nature and action of genes.

(2) *Somatic mutations as an embryological method.* In Section IV (6) it was mentioned that somatic mutations can be induced by X-raying fertilised eggs and larvae at various stages (Patterson, 1928, 1929; N. T.-R., 1929 *a, b, c*). Patterson has shown that X-raying *D. melanogaster* larvae at different stages of development produces somatic eye-colour mutations, resulting in eye mosaics with mutant areas of different size. The mutant areas are large if embryos or young larvae are rayed, and they are small if the larvae were older at the time of treatment. The reduction of the size of mutant areas with the raising of the age of the larvae at the time of treatment is shown in Fig. 11. Thus, the production of somatic mutations, especially

the use of the relatively frequent somatic chromosome mutations (in special genetic combinations, making them phenotypically detectable), can be used as an analytical method in embryology, allowing the study of the growth and differentiation of some of the *Organanlagen*. The production of somatic mosaics may also be of interest in connection with the question of interrelations between tissues of different genetic constitution (as studied by Sturtevant, 1932).

(3) *Comparison of the mutabilities in different species and races.* A really effective attack on this question can only be made with the help of radiation genetic methods. In comparing mutation rates in different species we must reckon with certain difficulties, especially with the "masking effects" already mentioned in Section

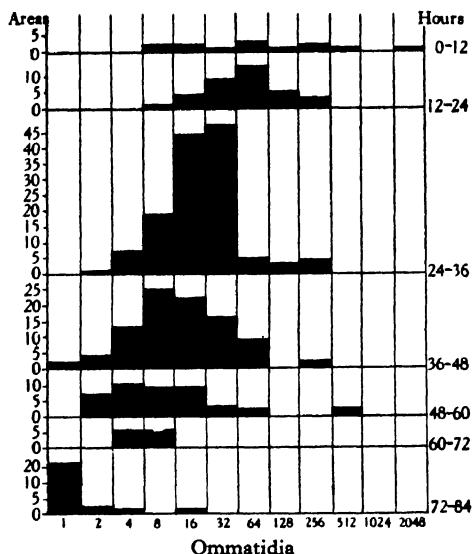


Fig. 11. The relation between the age (in hours) at which larvae of *D. melanogaster* were X-rayed and the number of ommatidia in the mutant eye spots (induced as somatic mutations of the white locus). (From Patterson, 1929.)

IV (5). But, if we take into account the different possible sources of errors, irradiation treatment can be used with success as a method in comparative genetics.

(4) *The study of the mutability of single individual genes.* X-ray and radium treatment is so far the only effective way of studying the mutational potencies of single individual genes.

In *D. melanogaster* the mutability of three sex-linked loci has been studied with the help of X-ray treatment. Dubinin, Serebrovsky, and their collaborators have studied mutations at the locus of scute which affect different groups of bristles and hairs on the head and thorax. Several dozen mutations were already induced at this locus and the phenotypic effects of different scute allelomorphs were compared. On the basis of these studies, the "theory of step-allelomorphs" was developed (Dubinin, 1929, 1930, 1932; Serebrovsky and Dubinin, 1930). The essential point of this hypothesis is the assumption of a complex structure of the genes. Different

parts ("subgenes" or "centres") of the gene affect different groups of bristles, and the various allelomorphs are mutations of different single "centres" or of neighbouring groups of "centres" of the same original gene. It is not the right place here to discuss the whole evidence for and against this hypothesis; in any case the induction of mutations at the locus of scute yields us a very interesting and important material for work upon the properties and structure of the genes.

Patterson and Muller (1930) and N. T.-R. (1932 *c*, 1933 *a*) induced many mutations by X-rays at the locus of forked in *D. melanogaster*. In this locus "direct" mutations (from the normal allelomorph to, or towards, forked) and "reversions" (from forked to, or towards, normal) are induced with about the same frequencies, showing the potential reversibility of at least some of the mutational changes,

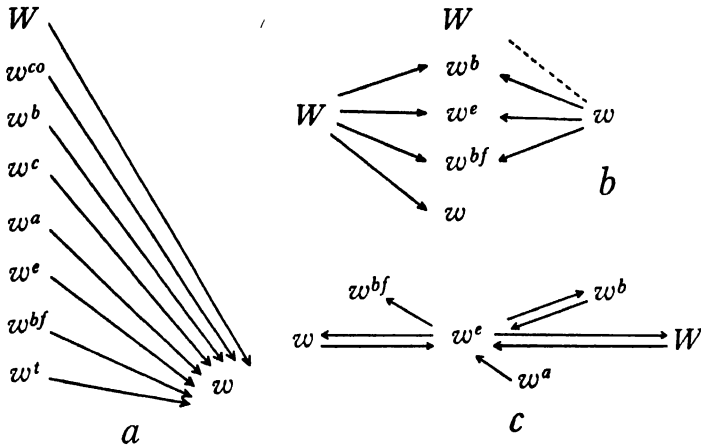


Fig. 12. X-ray induced mutations at the locus of white in *D. melanogaster*. *a*, The white allelomorph was induced from all other tested allelomorphs of this series; *b*, the allelomorphs blood, eosin and buff were induced as direct mutations from normal and as reverse mutations from white; *c*, all induced mutations from and to eosin.

being thus a serious objection against a generalisation of the "presence or absence" theory.

Special experiments have been performed on a large scale to study the mutability at the locus of white eye in *D. melanogaster* (N. T.-R., 1930 *b*, 1932 *a*, *c*, 1933 *b*, *c*). The rates of different mutational steps, induced by X-raying (with a constant heavy X-ray dose), were compared in flies containing different allelomorphs of this gene. As a result it was found that quite different mutations can be induced at this locus: "direct" and "reverse" mutations, mutations from one particular allelomorph to various others, and mutations from various allelomorphs to a particular one (Fig. 12). But the rates of the different mutations are different, some of the mutational steps being frequent and others very rare, thus showing a certain modal direction or "determinate variation" of the mutability at this locus. The results of these experiments are shown in Table XI. Another feature of these experiments was the finding of two otherwise indistinguishable normal allelomorphs of the white series,

differing both in the frequency and the modal direction of their mutability (N. T.-R., 1932 *a*, *c*, 1933 *b*, *c*). The different mutabilities of these two allelomorphs are shown in Fig. 3.

All the above experiments show that, in any case, the methods of radiation genetics can be applied with success to quantitative studies of the variability of individual genes. One of the most interesting problems arising in this connection is the experimental study of the evolution of the genes. We know that new allelomorphs can arise by mutation, and we must admit that, in the course of the evolution of a species, the number of the genes, and the profound properties of some of them (*e.g.* their general phenotypical effects and the direction of modal mutability) must also undergo changes. The Bar-eye mutation in *D. melanogaster* is probably a case in which a new gene arose in this species (Sturtevant, 1925). Some facts from the above-cited comparison of different scute mutations suggest that the genes scute and achaeta perhaps represent a stage in the differentiation of one original

Table XI. *Comparison of different mutation rates within the white eye series of multiple allelomorphs in D. melanogaster, produced by X-ray treatment (dosage 4800 r.). (From Timoféeff-Ressovsky, 1933 e.)*

Mutations	No. of flies	No. of mutations	Rates of mutations in $\% \pm m$	Differences of rates in $\% \pm m$ diff.
All direct	129,000	62	0.481 ± 0.061	0.436 ± 0.063
All reverse	134,500	6	0.045 ± 0.018	
$w^e \rightarrow w^{-e}$	39,000	15	0.385 ± 0.111	0.308 ± 0.119
$w^e \rightarrow w^{+e}$	39,000	3	0.077 ± 0.044	
$W \rightarrow w^x$	48,500	37	0.763 ± 0.125	0.708 ± 0.128
$w \rightarrow w^x$	54,000	3	0.055 ± 0.032	
$W \rightarrow w$	48,500	25	0.515 ± 0.102	0.254 ± 0.116
$w^x \rightarrow w$	80,500	21	0.261 ± 0.056	
$W \rightarrow w^x$	48,500	37	0.763 ± 0.125	0.393 ± 0.143
$w^{e-00} \rightarrow w^x$	73,000	27	0.370 ± 0.071	0.305 ± 0.078
$w^{-bf} \rightarrow w^x$	61,500	4	0.065 ± 0.032	

gene into two. And the finding of two normal allelomorphs of the white-eye series differing in degree of stability and modal direction of their mutabilities, indicates a differentiation of a gene (within the population of a species) in respect to its profound fundamental properties. The results of further experimental work in this direction will be of great interest, devoted to the search for allelomorphs (of the same gene) differing in direction and relative frequencies of mutation, and in the kind of characters affected by these mutations.

(5) *Practical applications.* The methods of radiation genetics can be practically applied in plant breeding. Most of the new mutations lower the viability of the organism and thus are, in most cases, of negative biological and economical value. But in certain combinations with other mutations, and in the presence of certain modifiers, even such mutations can reconstitute the normal viability of the wild type (N. T.-R., 1933 *d*), and thus have practical significance in plant breeding. The production of mutations *en masse* by X-rays or radium will have a special practical significance in those cases in which selection has already reached its limit, and in which crossing with related races or species must, for some reason, be avoided.

However, the practical significance of the induction of gene mutations is minimised by the fact that in the living populations of our crop plants and domesticated animals and of their wild relatives we have tremendous funds of still unutilised genes, which can be used in husbandry. But the induction of chromosome mutations will probably have unlimited applications, allowing of the "construction" of quite new karyotypes¹) in our cultivated plants.

In man radiation genetics has a purely negative significance: we must avoid any X-ray or radium treatments of the gonads (not leading to continuous sterility) in order not to accelerate the funds of injurious mutations already present in a rather high percentage in human (especially in European) populations. I believe slight treatments applied to many persons, performed without the control of good specialists, and without considering the danger of genetic injuries, to be most harmful in this respect. We must not forget that in *Drosophila* a general mutation rate of 1 per cent. (*i.e.* 1 mutation per 100 gametes) is produced by X-ray dosages of about 40–50 r. units²).

Conclusions. The following statements can be made concerning problems already solved in radiation genetics: (1) The genetic action of short-wave rays is a general one, capable of inducing all known types of mutations in all hitherto adequately tested objects. (2) The induced process of mutability shows far-going similarity and parallelism with the spontaneous one. (3) The induced rate of mutations is directly proportional to the dosage applied. (4) Within the range of X-rays the wave-length (if equal dosages are applied) has no specific influence upon the rate or the kind of induced mutations.

The following questions are not yet solved, or not yet decided with sufficient exactness: (1) The genetic action of ultra-violet rays; this question is of special interest since, theoretically, different ultra-violet rays could exert specific influences upon the germplasm. (2) An exact comparison of the influences of equivalent dosages of X- and γ -rays upon the process of mutation³. (3) The role of different accompanying factors and of different physiological conditions in the induction of mutations by short-wave radiations. (4) The intimate physical nature of the genetic action of radiations. (5) Various special genetic problems connected with the induction of gene and chromosome mutations (*e.g.* quantitative studies of mutability in different species, studies on the direction of mutability and on "evolutionary potencies" of single individual genes, studies on the mechanism of chromosome mutation, etc.).

Soon after the first discovery of a pronounced action of X-rays on the rate of mutation, Muller himself and several other biologists expressed the idea that the origin of spontaneous mutations could perhaps be ascribed to "natural radiations"

¹ The "karyotype" is the number and form of the chromosomes typical of a given species (Levitsky, 1924).

² The calculation of the dosage producing a general mutation rate of 1 per cent. is based on the following data: 3000 r. produce about 10–15 per cent. sex-linked mutations; the genetically active part of the X-chromosome constitutes about one-fifth to one-sixth of the whole set of chromosomes in *D. melanogaster*, and the mutability of the autosomes is as intensive as that of the X-chromosome; the general rate of mutations produced by 3000 r. is, accordingly, about 60–75 per cent.; a mutation rate of 1 per cent. is thus produced by 40–50 r. units.

³ See footnote 2 on page 424.

present in the environment (Muller, 1928; Babcock and Collins, 1929; Hanson and Heys, 1930; Joly and Dixon, 1929; Olson and Lewis, 1928; N. T.-R., 1929; Tschetverikov, 1929). But calculations, carried out independently by Muller and Mott-Smith (1930), N. T.-R. (1931 *a*), and Efroimson (1931), show that the amount of "natural radiation" is insufficient to account for the rate of spontaneous mutations. Muller and Mott-Smith estimated that the actual amount of natural radiation is 1333 times too low, N. T.-R. estimated that it is 462 times too low, to produce the observed rate of spontaneous mutations. I mentioned the possibility (N. T.-R., 1929 *d*) that the concentration of radioactive substances in the living organisms, discovered by Vernadsky (1929, 1930), could account for at least some of the spontaneous mutations. But it is clear that the small amounts of radioactive substances contained in living matter cannot account for all spontaneous mutations. We must thus search for other sources of factors inducing mutation, within the organisms and in the environment.

V. HEAT AND OTHER TREATMENTS AS CAUSES OF MUTATION.

Most of the genetic experiments hitherto performed on the production of mutations with agents other than X-rays or radium either do not fulfil the requirements detailed in Section III, or they have given doubtful results. Many of them will therefore be omitted, or only mentioned briefly, below.

(1) *Temperature experiments.*

We will omit a discussion of older work and concentrate our attention on modern genetic experiments using temperature as agent for inducing mutations. The whole problem can be divided into two distinct fields of research, connected with two different methods of treatment: (1) the study of the influence upon the rate of mutation of different temperatures lying within the "normal physiological temperature scale" for the given organism, and (2) experiments with "temperature-shocks," *i.e.* treatment for a short time with extreme temperatures, having a sublethal or substerilising action.

Experiments within the range of normal temperatures. As early as 1919 Muller, after having found methods of determining the normal rate of spontaneous mutations in *Drosophila*, published the results of his first temperature experiments (Muller and Altenburg, 1919). Flies kept at higher gave somewhat more mutations than flies kept at lower temperatures. But this result was inconclusive, the difference in the rates of mutation being statistically insignificant. Further experiments, published 1928, gave substantially the same results (Muller, 1928 *b*): flies reared at 27° C. showed about three times as many mutations as those kept in 19° C. Unpublished experiments of N. T.-R. (1927-30) confirmed the results obtained by Muller: at 25° C., the flies gave about three times as many mutations as at 15° C. Taken all together they give a statistically quite significant and conclusive result; the spontaneous rate of mutation is directly proportional to the temperature, and the rate of mutation is tripled by an increase of temperature of about 10° C. In other words, the spontaneous rate of mutation follows the Van't Hoff rule.

This latter conclusion, suggesting that the spontaneous rate of mutation behaves as an ordinary multimolecular chemical reaction, is of great interest in connection with the results of radiation genetic experiments. One of the general conclusions which can be drawn from radiation genetics is that mutation belongs to the type of monomolecular reactions, not following the Van't Hoff rule. Experiments of Muller, Stadler and N. T.-R. have shown that different temperatures applied during irradiation have no influence upon the induced rate of mutation (Section IV (5)), thus leading to the same conclusion that mutations are monomolecular reactions. Most of the X-ray induced mutations are identical with spontaneous ones; and the same individual mutational event (the change of a definite allelomorph, leading to the formation of another definite allelomorph) cannot possibly be in one case a monomolecular and in another a multimolecular reaction. Thus there seems to be a discrepancy between the results of radiation and temperature experiments. But this discrepancy disappears if we assume that in the temperature experiments certain sources of mutation-inducing factors, and not the mechanism of the mutation event itself (which is monomolecular) follow the Van't Hoff rule. At the end of the preceding section (IV (8)) we had already reached the conclusion that some internal sources of mutation-inducing factors must exist. We do not know what they are; perhaps some processes of chemoluminescence (subject to the Van't Hoff rule) take place within the organism, and so constitute factors inducing mutation. At present any theorising in this direction is useless; only further experimental work will show whether this or some similar hypothesis conforms with the empirical facts.

Experiments with temperature shocks. This method consists in treating the organisms with "substerilising dosages" of temperatures (high or low), lying beyond the limits of normal physiological conditions.

In *D. melanogaster* this method of treatment was first applied by Muller (1928 *d*). He treated adult males with substerilising dosages of 36° C. (40–60 hours) and mated them to *CIB* ♀♀. The treated series gave a slight, statistically insignificant, increase of lethal mutations. Similar experiments (adult ♂♂ treated at 37° C. for a period of 20 hours) were made independently, and at the same time (1927–8), by N. T.-R. (unpublished); they gave substantially the same results. The same treatment was applied on a large scale by Muller and Mackensen in 1932 (exhibited at the Sixth Intern. Congr. Genet.) and also gave only a very slight increase of the rate of lethal mutations. The results of these experiments are summarised in Table XII.

In the preceding experiments adult males were treated. N. T.-R. also applied the same treatment (1927–8, unpublished) to old larvae: 5–6 days old larvae were subjected to a temperature of 37° C. for about 15 hours, and the hatching males were mated to *CIB* ♀♀. These experiments gave negative results: the rate of lethal mutations showed no significant increase. Efroimson (1932), using a similar method of treatment, got a slight, but statistically significant increase of the rate of lethal mutations in the treated series.

Goldschmidt (1929) treated *D. melanogaster* larvae (5 days old) with 37° C.

(12 hours). The hatching flies were crossed *inter se* (in most cases in mass cultures) and inbred for three generations. In some of the treated series many visible mutations (affecting eye and body colour, wings, bristles, etc.) were found in F_1 and F_2 . The most striking finding in these experiments was the fact that almost all the mutations appearing in F_1 and F_2 were recessives, and a part of them even autosomal recessives. If contamination and segregation of mutations already present in heterozygous condition in the original cultures are excluded, this finding can only be explained by the assumption of a very pronounced specific action of the agent upon certain genes. An autosomal recessive mutation must be induced *en masse* in order to have a chance of appearing in F_2 or even in F_1 . This is the conclusion drawn by the author from his experiments (Goldschmidt, 1929). Substantially the same method of treatment was used by Jollos (1930, 1931 *a, b*, 1932) in his experiments on induction of mutations in *D. melanogaster*. Jollos drew the conclusion that temperature shocks, if applied to subsequent generations, induce (in

Table XII. *Experiments on the effect of heat treatment of adult males of D. melanogaster on the rate of sex-linked mutations. (Muller, 1928 d, 1930 a and unpublished data of Muller and Timoféeff-Ressovsky.)*

Author and date of experiments	Analysed chromosome	Treatment	Series	No. of cultures	No. of mutations	Percent. of mutations	Diff.
Muller, 1928	X-chromosome (CIB method)	W series: adult ♂♂ treated with 35–36° C. for 24–40 hours. C series: untreated controls	W	493	4	0·81	1·2
			C	482	2	0·41	
Mackensen and Muller, 1932			W	5952	24	0·40	3·4
			C	5887	10	0·17	
N. T.-R. 1927–28		W adult ♂♂ in 37° C. for about 20 hours. C untreated controls	W	758	3	0·39	0·8
			C	617	1	0·16	

certain genes) “determinate mutation,” proceeding step by step from the normal allelomorph towards the extreme mutant allelomorph. In his last paper (Jollos, 1933) he describes the results of some of his experiments showing that slight differences in the method of treatment (moist or dry heat) cause different specific effects on mutability (specific induction of different mutations). He thus draws the conclusion that the process of mutation can be directed by will, using slight modifications of the heat treatment.

The experiments of Goldschmidt and Jollos raise a number of most interesting questions. But, unfortunately, they do not solve them. In these experiments the most important question, namely that of the quantitative mutation-inducing action of heat treatment, was not adequately analysed. Moreover, all similar experiments performed in other laboratories have given results which are quite different from those of Jollos. Experiments by Rokizky (1930), Ferry (Ferry, Shapiro and Sidoroff, 1930), Redfield and Schultz (1931, demonstrated at the Sixth Intern. Congr. Genet. 1932), Demerec (*ibid.*), Sturtevant (*ibid.*) and Plough (Plough and Ives,

1932) are summarised in Table XIII. None of them have given results similar to those of Goldschmidt and Jollos. A slight increase in the rate of mutation, following heat treatment, was obtained by Plough; but, although he treated eight subsequent generations of flies, no mutation *en masse* or "determinate mutation" of certain genes could be observed. Similar results have recently been obtained by Grossman and Smith (1933) and in unpublished experiments of N. T.-R.; in the latter experiments the *attached-X* method of crossing was used and an exact determination of the rate of sex-linked mutations was made.

Thus it seems that in *Drosophila* the genetic effect of temperature shocks is non-specific and not at all so pronounced as the effects of radiation treatment.

Table XIII. *Experiments performed to test whether heat treatment (37° C.) of larvae (3-6 days old, for 12-24 hours in heat) increases the rate of mutations in D. melanogaster. (From exhibits at the Sixth Intern. Congr. Genet.)*

Authors and date of experiments	Treatment	Generations	Series	No. of cultures	No. of flies	No. of mutations
Rokitzky, 1930	After Goldschmidt	F_1-F_3	Treated Control	— —	15,147 2,731	5 1
Ferry, Shapiro and Sidoroff, 1930	After Goldschmidt	F_1-F_3	Treated Control	265 62	11,771 2,590	0 0
Redfield and Schultz, 1931	After Goldschmidt	F_1-F_3	Treated Control	359 62	38,025 27,379	5 2
Demerec, 1931	After Goldschmidt	F_1-F_3	Treated Control	215 37	33,305 8,176	1 0
Sturtevant, 1932	3-5 days old larvae several hours in 37° C. or intermittent in 37° and in 4-10° C.	F_1-F_3	Treated Control	— —	39,098 —	2 —
Plough, 1932	6 days old larvae for 24 hours in 37° C.	F_1-F_8	Treated Control	580 236	110,000 55,000	18 2

In plants, treatment with temperature shocks was applied to *Antirrhinum* by Baur (1930) and Stubbe (1930, 1932). These experiments gave negative results.

(2) Experiments with other agencies.

Almost all experiments in which agents other than radiation or temperature are used involve special difficulties. In most cases we do not know whether the treatment reaches the germ cells, and, if so, in what form it reaches them.

Chemical treatments. We will not here discuss the older literature, since it does not fulfil the requirements of exact experimentation. More recently Harrison and Garrett (1926) reported that melanistic mutations were produced *en masse* in butterflies (*Selenia*) by lead and manganese added to the food. But certain recent experiments (Lycklama and Nijeholt, 1932), and the extraordinary high mutation rate obtained by Harrison and Garrett, together with some theoretical considerations

(the high concentration of melanistic mutations in wild populations), render it improbable that all of these mutations were really induced by the treatment. Further experiments, using carefully selected and inbred material, must in any case be made before the question of chemical induction of mutations in butterflies can be decided.

Experiments on the induction of mutations in mice by alcohol treatment have been performed in many different laboratories. But these experiments have not yet yielded any conclusive results. Even the authors who believe that mutations can be induced by alcohol, and at the same time perform exact experiments on a large scale (*e.g.* Bluhm, 1930), cannot prove the existence of hereditary effects of alcohol treatment in an objective and conclusive way.

Extensive and exact experiments have already been performed with *Drosophila*, using different chemical treatments as mutation-inducing agents. Morgan tried the effects of ether and alcohol (1914) with negative results. Mann made extensive experiments on the effects of alcohol, arsenic, quinine, morphine, methylene blue, lead, lithium and copper as agents for inducing mutations. All these experiments gave negative results (Mann, 1923). Muller treated *D. melanogaster* with semilethal concentrations of lead acetate (1 per cent. of the food), arsenic trioxide (0.015 per cent. of the food) and manganese chloride (0.62 per cent. of the food) throughout the whole life cycle (Muller, 1928 *d*, 1929, 1930 *a*). None of these agents showed any influence on the rate of mutation. Muller also made experiments using Janus green (0.25 per cent. of the food, throughout the whole life cycle). These experiments, performed on a large scale (1058 F_1 - F_2 *CIB* cultures from treated males and 1013 untreated control cultures) also gave negative results (Muller, 1930 *a*). Ssacharoff (1932, 1933) has recently published his results on treating *D. melanogaster* eggs with iodine in potassium iodide (for 2 min.). The treated series gave rather more mutations than the controls, but the difference is statistically insignificant.

Thus, all experiments hitherto performed on chemical treatments of *Drosophila* have given negative, or (as in the case of iodine treatment) inconclusive, results. Much further work must be done to find out whether chemical treatments exert an influence upon mutation in *Drosophila*. The most interesting feature of such experiments would be the discovery of a specific, differential, action of a certain agent upon certain definite genes. And the chief difficulty of these experiments will be the discovery of methods which would enable the agents applied to penetrate into the chromosomes of the gametes of the treated organisms.

The chemical treatment of plants is technically easier. Here the method of chemical treatment of the seeds can easily be applied, the latter being a physiologically rather resistant stage of the plant. The seeds can either be treated with solutions or they can even be centrifuged in these solutions, in order to accelerate the process of penetration of the chemicals.

Exact experiments on production of mutations in plants by chemical treatment were first made by Stadler (1928 *b*). He found that the impregnation of barley seeds with salts of heavy metals (barium, lead and uranium nitrates) has no

influence on the rate of mutation. Baur (1930, 1932) and Stubbe (1930) treated seeds and seedlings of *Antirrhinum majus* with various chemicals (such as salts of heavy metals, alcohols and acids), applying the method of centrifugation in some of the experiments. The results were inconclusive, although the percentage of mutations is perhaps somewhat higher than in the controls. The methodological mistake made in these experiments is the simultaneous treatment with several dozen different agents; it is quite evident that under these circumstances every single series yields statistically insignificant results.

Other treatments. Morgan (1930) performed extensive experiments to test whether a heat injury to the eyes of *D. melanogaster* is inherited, or, at least, has some effect on the rate of mutation. This treatment is followed by a permanent, deep red, colouring of the Malpighian tubules of the treated flies. Such flies, and also untreated controls from the same stocks, were inbred. These breeding experiments gave negative results; no inheritance of this acquired character, no specific eye defects, and no detectable increase of the general spontaneous rate of mutation, was found in the progeny of treated flies. These experiments were repeated by Muller (1930 a); he used a method of crossing suitable for the detection of induced lethals. The results of Muller's test were also negative.

It has already been mentioned that *Drosophila* experiments have been made to test the influence of supersonic waves and of electricity on the rate of mutation. Hersh, Karrer and Loomis (1930) found (as a result of experiments performed on a very large scale) that sublethal doses of supersonic waves do not influence the rate of mutation. Experiments of Horlacher (1930) and of Schmitt and Oliver (1933), carried out independently and using somewhat different methods of treatment, showed that electricity has no influence on the rate of mutation in *Drosophila*.

VI. GENERAL CONCLUSIONS.

The above review of all experiments hitherto performed on the induction of mutations leads to the following conclusions.

The treatment with short-wave radiations and high-speed electrons (X-rays, γ -rays, β -radiation) is so far the only effective method of inducing mutations, giving constant and measurable results. It is almost certain that radiation treatment is capable of inducing hereditary changes in all organisms, since quite different plants and animals hitherto tested have given substantially the same positive results. The power of X-rays and radium to induce all known types of heritable variations makes the application of the radiation genetic methods most valuable for analytical genetic studies, for instance, in comparative genetics of related species, in quantitative studies of the mutabilities in different species and of different individual genes, in cytogenetics, in "genetic engineering" (*i.e.* in the synthesis of new genotypes and races).

We have good reasons to believe that, besides those genetic problems which have already either been solved or attacked by radiation genetic methods, in the future the solution of the most fundamental problems concerning the nature of the genes and of gene changes will be connected with radiation genetics.

All other treatments hitherto applied have given no definite nor conclusive results. Nevertheless, temperature experiments and some of the chemical treatments show that further experimentation will yield important results.

One of the most interesting future problems is the discovery of methods of treatment which will work differentially and enable us to induce at will certain types or groups of mutations. But only experiments, using thoroughly prepared, genetically pure, material, and adequate methods of treatment and breeding, will bring us further towards the solution of these important biological problems.

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CONCERNING THE EVOLUTION OF THE CEPHALOPODA

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IN vol. 8 of *Biological Reviews* (1933) L. F. Spath published an article on "The Evolution of the Cephalopoda," in which he strongly criticises my investigations in this field (1920-32). In many points our views are in full agreement, but we differ entirely as to the phylogeny of the Nautiloidea, the derivation of the Goniatitoidea, and the radicle of the later Ammonoidea.

Because of this criticism I have recently undertaken a revision of the material of my former investigations and have examined a great number of new sections and other preparations of Ammonoidea, as well as Nautiloidea. The results of these investigations are set forth in two papers "Bau und systematische Stellung der Gattung *Volborthella* Schm." and "Zur Stammesgeschichte der Cephalopoden," each illustrated by several plates, which are now in press in the *Palaeontologische Zeitschrift*, vol. 16, and in the *Jahrbuch der Preuss. Geol. Landesanstalt*, vol. 55, respectively. I have tried to show that the different opinions held by Spath and myself are due partly to several errors on his part concerning the Paleozoic ammonoids and partly to his classificatory method of emphasising the external morphological characters and neglecting the internal features as well as the ontogenetic development.

My investigations concerning the evolution of the Cephalopoda have led to the following principal conclusions contrary to those arrived at by Spath:

1. All available characters of the organisms must, of course, be used as a basis for classification and for elucidating phylogeny, but they are of different ranks. As to their classificatory and phylogenetic significance, there is a hierarchy of characters resembling that in the scale of classification. Each differential diagnosis of the successive systematic units can be based therefore only on a single or on a few decisive characters selected from the mass of all those available.

2. In general, the most important are considered to be the *internal*, often minute, characters and their developmental features, because they are more independent of the mode of life than the external ones.

3. Ontogeny is thought to be a valuable help in recognising the general steps of ancestral phylogeny, though there are *modi* of development other than purely palingenetic.

4. *Volborthella* is undoubtedly a primitive cephalopod. It has no connection with *Salterella*.

5. In the Nautiloidea, as well as in the Ammonoidea, we have a general evolutionary trend from straight or slightly curved to closely coiled forms. Secondary uncoiling is only a rare and exceptional process.

6. *Bactrites* is a true representative of the ammonoids and not merely an *Orthoceras* with a marginal siphuncle.

7. The earliest goniatites differ in nearly all characters from the more or less imperforate nautilicone nautiloids (e.g. *Barrandeoceras*). Their derivation from the latter is held to be impossible.

8. *Lobobactrites* and *Gyroceratites* are not descendants of coiled goniatites; they are primarily straight and loosely coiled respectively.

9. Evolution of the early goniatites proceeds from straight to coiled. Stages in this evolutionary series are *Bactrites*, *Lobobactrites*, *Gyroceratites*, *Mimagoniatites*, *Agoniatites*, *Anarcestes*, *Werneroceras*, etc. The new genus *Anetoceras* (genotype: *A. arduennense* (Stein.)) from the Lower Devonian of Germany, characterised by a *Crioceras*-like coiling of the whorls, is assumed to be a further example of this trend. The primary radicle of all these forms is seen in the orthoconic nautiloids.

10. In the goniatites, the siphuncle beginning with the first septum has a stable position at the external (ventral) side of the volutions. There are no goniatites with unstable siphuncles.

11. Certain Permian and Mesozoic ammonoids show ontogenetically a gradually accelerated displacement of the siphuncle from an originally internal (dorsal) or intermediate position to an external (ventral) one.

12. I see no possibility of deriving these forms from the goniatites with their constantly ventral position of the siphuncle. It seems to me that their radicle must be sought amongst the clymenids having a dorsal siphuncle.

13. Only those ammonoids without displacement of the originally ventral siphuncle, to my mind, may have arisen from the goniatites.

A detailed discussion of these statements is contained in the two papers mentioned above.

GENES AND INDUCTORS OF SEX DIFFERENTIATION IN AMPHIBIANS¹

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CONTENTS.

	PAGE
I. Introduction	460
II. Geographical variation, sex races and the evolution of gonochorism	461
III. The genes	463
IV. The inductors	469
V. Discussion	480
VI. Summary	485
References	487

I. INTRODUCTION.

ONLY ten years ago the occurrence of sex reversal in amphibians was still a contested matter. To-day, this process has become so well known that it could no longer claim the special attention of biologists if it were not for its bearing on more general problems, particularly those of genic action and embryonic induction.

Work by many investigators on insects, culminating in the breeding experiments with *Lymantria* and *Drosophila*, has provided a clear insight into the hereditary mechanism which almost unfailingly rules over sex determination in organisms of the type that we may classify as *sex chromosome-gonochorists*². So splendid was this success as to distract, temporarily, the attention of biologists from the fact that sex determination fundamentally is an embryological process, and that in hermaphrodite plants and animals the determination of male and female embryonic areas is independent of chromosomal or genic segregation.

Experiments with *Lymantria* and *Drosophila* furnish ample evidence that even gonochorists are amphisexual in constitution, and that they follow their normally unisexual course of development only through suppression of one sex potentiality. Prevalence and suppression are determined by the asymmetrical (unbalanced) distribution of male and female genes through the sex-chromosome mechanism. Upon this discovery are founded the quantitative theories of genic action (Goldschmidt) and genic balance (Bridges), which have become so useful in the analysis of phenomena of sex inheritance in all animals and plants. However, the application of these

¹ This report includes many results of so far unpublished investigations of the author, which have been aided by grants of the Committee for Research in Problems of Sex of the National Research Council.

² The term *gonochorism* was introduced by Haeckel to designate the condition in which ovaries and testes are in separate individuals (males and females), as opposed to the condition of *hermaphrodism* or co-existence of male and female sex glands in the same individual.

principles to the case presented by amphibians soon brings out the fact that *Lymantria* and *Drosophila* represent only one special type of sex differentiation. Generalisations which are derived exclusively from this insect material must of necessity be afflicted with certain insufficiencies, mainly for the following reasons. The insect work provides no clue as to the possible origin of the sex-chromosome mechanism. Such information can more likely be expected from the amphibian group where the frequent instances of hermaphroditism and sex reversal clearly indicate that the chromosome mechanism of sex determination is not yet fully established. Moreover, while it is generally assumed that the gene is connected with its ultimate phenotypical product by a chain of intermediate reactions, the insects have proved a most unyielding material in attempts to gain an insight into the nature of this process of genic action. Here again the amphibians offer more favourable conditions, since the inductive processes controlling morphological sex differentiation are open to experimental investigation.

II. GEOGRAPHICAL VARIATION, SEX RACES AND THE EVOLUTION OF GONOCHORISM.

Pflüger (1881) was the first to describe the so-called juvenile hermaphroditism of the males of certain local races of the grass frog (*Rana temporaria*). The case may be illustrated with the race from Freiburg (Table I). At the time of metamorphosis (April) all animals (from eggs fertilised in early March) are found in the female phase. During the second half of the summer, 50 per cent. undergo sex reversal. Passing through a stage of morphological hermaphroditism they change into males (Witschi, 1929c). There exists also an adult hermaphroditism of the females. Adult hermaphrodites of frogs have often been described (recent review by Cheng, 1933). Crew (1921) and the author (1923a, 1929c) have analysed by cross-breeding four of these hermaphrodites which cover the entire range from a prevailing female to the almost completely male morphological condition. Two specimens furnished fertile eggs and sperm; the remaining two yielded sperm only, the eggs being in a degenerate state. All four proved to be genetical females. Adult hermaphrodites of the grass frog therefore are females changing to a male phase. Among fifty "females" collected near Freiburg three were in the process of sex reversal. This high percentage indicates that juvenile and adult hermaphroditism are correlated characteristics of the Freiburg race. Strictly speaking, we do not here have males and females, but rather two classes of protogynous hermaphrodites, one with a short and the other with a long female phase.

Table I. *Sex conditions in first-year frogs from Freiburg (Witschi, 1930a).*

Time	Females %	Hermaphrodites prevailingly female %	Hermaphrodites prevailingly male %	Males %	Total number of animals preserved
April	100	—	—	—	515
July	62	10	28	—	150
August	52	2	22	24	209

At other places (Berlin, Bonn) the hermaphrodite features are less pronounced. The female phase in the young males is shorter, so that at the time of metamorphosis one finds the transformation already under way.

Finally, in the Alps (Davos) and in north-eastern Europe (Riga, Koenigsberg), the hermaphrodite character has practically disappeared. Males and females differentiate as separate sexes at an early larval stage.

It goes without saying that the juvenile hermaphroditism is far more helpful in establishing racial types than the adult hermaphroditism, since in our laboratories frogs cannot easily be raised in large numbers to the adult stage. I therefore use the sex ratio at metamorphosis to characterise the racial type (Witschi, 1914, 1929*c*). If at that stage a breed consists of females only, the type is designated *undifferenti-*



Fig. 1. Map of Europe showing distribution of sex races of *Rana temporaria* ●, differentiated type, ○, semidifferentiated type, ○, undifferentiated type 0° C isotherm of January, ---- 20° C isotherm of July; .. northern limits of the range of wheat, beech and vine (After Witschi, 1930*a*)

ated; if males and females are present in equal numbers *differentiated races* are spoken of. Between these two extremes are scattered a series of *semidifferentiated races* in which from 50 to 90 per cent. of the animals are females while the rest are hermaphrodites or hermaphrodites and males.

The significance of these racial differences in connection with the problem of the evolution of gonochorism becomes still more emphasised by the interesting relationships found in their geographical distribution. The accompanying map (Fig. 1) shows that the differentiated race has been found in two widely separated regions, the Alps and the Baltic countries, both of which are characterised by long and severe winters. On the other hand, the undifferentiated races inhabit regions of a moderate climate. It is important to note that frogs from the Alpine valleys of Bavaria, Davos and Berne, though geographically separated, all belong to the same sex type. These Alpine as well as the Baltic local groups no doubt branched off in

post-glacial times from those living in the temperate regions of France, Holland, Germany and Poland, which are now of the undifferentiated or semidifferentiated type.

Geneticists are generally inclined to assume that geographical races arise by undirected mutations and that climatic and other geographical factors play a part only by favouring survival of different mutations in different regions. However, the comparative study of diverse animal and plant groups reveals an evolutionary trend from hermaphroditism to gonochorism that exhibits a distinctly orthogenetic character. Its direction rests on inherent factors and is independent of selection; the environment merely influences the speed of the evolutionary progress.

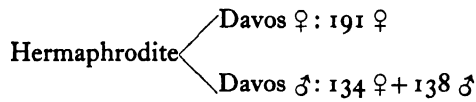
It is certain that sex variations like those described for *Rana temporaria* occur also in other amphibian species. Hertwig (1912) worked extensively with *R. esculenta*, Swingle (1921, 1926) reported on sex races in the bull frog (*R. catesbiana*), Humphrey (1931*a*) and Witschi (1933*c*) have established local variations of similar type in *Amblystoma maculatum*. However, in none of these species has the geographical distribution been studied sufficiently to throw further light on the relationship of climate and sex type. Since hermaphroditism in the grass frog is most pronounced in regions of temperate climate, we may expect it to occur relatively frequently among tropical amphibians. The only contribution of importance to this subject is the paper of Roxas (1929), according to which hermaphroditism seems to be very common in the Philippine frog, *Rana vittigera*.

III. THE GENES.

The analysis of the mechanism of sex inheritance in frogs is of interest because of its bearing on the problem of the evolution of the sex-chromosomes. As outlined above, the frogs occupy some of the steps-between true hermaphrodites and true gonochorists. The former are homozygous with respect to any possible sex-determining genes (**MMFF**) and have an even number of chromosomes. No animal or plant hermaphrodite is known to be an exception to this rule. With the evolution of the gonochorists, different genetical mechanisms of sex determination have arisen. Here we are interested only in the sex-chromosome type, which is characterised by the loss of one chromosome in the digametic sex. Cytologically, the *XY* and the *XO* conditions mark two main phases in its evolution. The study of the frogs now supplies evidence that in the *Y*-chromosome the sex gene, which is the allelomorph of **F** in the *X*-chromosome, does not drop out by a single mutation, for it plays a distinct rôle as a quantitatively reduced female gene (**f**). This *Y f* is still a relatively potent factor in undifferentiated races, but has a decidedly lower value in the differentiated races. The finding of the **f** gene (Witschi, 1914*b*) was the first evidence of any hereditary factor localised in a *Y*-chromosome. The general formulae for sex genes in frogs appear now as **MMFF** (female) and **MMFf** (male), with **M** factors located in autosomes, **F** in *X*-chromosomes and **f** in *Y*-chromosomes.

The sex gene of the *Y*-chromosome is evidently not the only one to undergo quantitative variation. Applying the principles of Goldschmidt and Bridges, *i.e.* by

From a first series we give only the result of an outcrossing of one hermaphrodite with a pair of differentiated race from Davos:



The sex ratios among the two groups of offspring show clearly that the female sheds only one type of eggs (**MF**), while the male produces two kinds of sperms: *gynosperms*, carrying an excess in female genes (**MF**), and *androsperms*, with an excess in male genes (**Mf**). It is in conformity with this and with other work on sex inheritance that we write the genetic formulae **MMFF** for female and **MMFf** for male frogs.

The second series gives the results of crosses between females of semidifferentiated race and males of the three different racial types. In other words, the eggs are of the same kind in all three combinations and the differences are due to the genes introduced by the sperms. Since the gynosperms produce 50 per cent. of females in every combination it is evident that more precisely the differences are caused by the androsperms only (Table III, last column). The androspERM of undifferentiated races produces offspring with an extended juvenile female phase; the hermaphrodite feature is much reduced in the second combination and is practically absent in the last one. On this type of evidence is based our conclusion that a female factor (**f**) carried in the Y-chromosome has a relatively high value in the undifferentiated races, becomes diminished in semidifferentiated races and reaches its lowest value in differentiated races. Anyone hesitating to admit the existence of the **f** gene of the Y-chromosome would have to assume diminished autosomal **M** genes in semi- and undifferentiated races. This, however, would imply that females of differentiated races should exhibit more hermaphrodite tendencies than those of undifferentiated races, which is in conflict with the observed facts.

Table III. *Combination of sperms from three racial types of frogs with eggs that came from the same females in all three cases (Witschi, 1929c). These parent females were of semidifferentiated race. Sex condition of offspring established at the metamorphosis stage.*

Parent male		F ₁ offspring		
Origin	Racial type	Total	Egg + gynospERM	Egg + androspERM
Freiburg	Undifferentiated	789 ♀ 19 ♂	404 ♀ (50 %)	385 ♀ 19 ♂ (48 %) (2 %)
Berlin	Semidifferentiated	478 ♀ 75 ♂ 250 ♂	401 ♀ (50 %)	77 ♀ 75 ♂ 250 ♂ (10 %) (9 %) (31 %)
Davos	Differentiated	353 ♀ 11 ♂ 342 ♂	353 ♀ (50 %)	11 ♂ 342 ♂ (1.5 %) (48.5 %)

The third series (Table IV) is chosen from Hertwig's (1912) experiments with *R. esculenta*. It is a peculiarity of undifferentiated races of this species that the

oocytes enter the stage of rapid growth and yolk formation relatively late and that consequently the ovaries as a rule remain small throughout the first year (Fig. 2a). The offspring of undifferentiated races at the metamorphosis stage consists exclusively or prevailingly of animals with this type of gonads: 100 per cent. (♀), while the offspring of differentiated races consists of males and of females with large ovaries: 50 per cent. ♂ + 50 per cent. ♀ (Table IV, combinations 1 and 6). We consider the slow ovarian development as an expression of the lower potency of the female genes in undifferentiated races, or rather of the smaller difference in male and female factors. In this series is also included a male of Italian origin which is of a more highly differentiated type than any of the German males. Examination

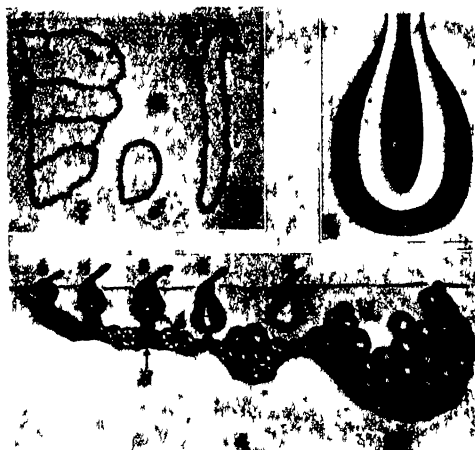


Fig 2a Sex glands of three specimens of *Rana esculenta* slightly over one year old ♀, ovary of female of differentiated race, ♂, testis of male of differentiated race, (,) ovary-like gonad of either sex of undifferentiated race (After Witschi, 1929d)

Fig 2b Diagram illustrating the localisation of the male (M) and female (F) inductors in the gonads of the higher vertebrates (After Witschi, 1914)

Fig 2c Longitudinal section through the gonad of a larval female of the toad 1-5, rete cords, A, primary gonad cavity or albuginea, S, abortive sex cord. The cortex is highly developed in the anterior region where it forms the so-called Bidder's organ (Bo) (After Witschi, 1932)

of the four combinations 3-6 in Table IV shows that all eggs fertilised with androspersms give typical males, which is the expected result since both parent males are of differentiated type. However, the comparison of groups 3 and 5 shows a characteristic difference in the offspring from eggs fertilised by gynospersms, indicating

Table IV. *Cross-breeding in Rana esculenta* (Hertwig, 1912). ♀ u, ♂ u, female and male of undifferentiated race; ♀ g, ♂ g, female and male of German differentiated race. ♂ i, male of Italian differentiated race.

	♂ u			♂ g		♂ i	
♀ u	1 ♀	64 (♀)	3 ♂ ¹	69 (♀)	54 ♂ ²	50 ♀	52 ♂ ²
♀ d		109 (♀)		34 ♀	52 ♂ ²	142 ♀	140 ♂ ²

that **F** of the Italian male has a higher value than **F** of the German male (compare also Table II). Comparing now groups 3 and 4 we notice a similar difference, which must, however, in this case be due to the difference in the factors **F** carried by the eggs of the two parent females (see Table II). This set of cross-breeds therefore brings out the fact that the female-determining genes (**F**) of the *X*-chromosomes have different quantitative values in various sex races of *R. esculenta*. In *R. temporaria* similar differences exist, though they are more conspicuous among adult than juvenile females.

The occurrence of quantitative variations lends itself to the interpretation of the sex genes as aggregates of smaller units. "Subgenes" could either be located close together and still act as a single "one-point gene," or else they might be distributed along the carrier chromosome. The latter arrangement would open the possibility of crossing-over playing a rôle in the evolution of sex-chromosomes. On this basis the writer has interpreted some breeding experiments on the fish *Aplocheilichthys* by Aida (Witschi, 1932), and the same principle may possibly apply to the results of self-fertilisation of hermaphrodite frogs. Since hermaphrodite frogs are genetical females, one would expect an entirely female offspring from self-fertilised eggs. We obtained, however, in a first case $46 \text{ } \varnothing + 2 \text{ } \sigma$, and in a second instance $144 \text{ } \varnothing + 20 \text{ } \sigma$. If one assumes that the "subgenes" of **F** were differently arranged in the two *X*-chromosomes of the parent, then crossing-over may have produced a few chromosomes of very low value. Selfing simply would give a chance of combining such extremely low values. However, we still prefer to think that the unexpected juvenile hermaphrodites are due to peculiar physiological factors which are responsible also for the observed partial self-sterility, excessive rate of mortality during cleavage, and teratological development of many embryos in the cultures of self-fertilised eggs (Witschi, 1929c, p. 205). One must always take into consideration that sex in frogs is in a labile state and easily influenced by factors other than sex genes. Cross-breeding of two hermaphrodites would settle this matter.

In favour of a linear arrangement of subgenes are the experiments of Dobzhansky and Schultz (1931) who have shifted the type of intersexes toward femaleness by the addition of fragments of *X*-chromosomes. We are hesitant, however, to follow Bridges (1932) in his conclusion that sex depends "upon a host of genes which differ in degree and character of their action," or even more broadly expressed in his formulation of "genic balance," that "...each character or feature of the adult is produced by the joint or cooperative action of all the genes of the entire set...." Such statements will probably have to be considered as philosophical ideas rather than generalisations of established facts, or else they express only the common experience that every single character, during its development, is exposed to and may be influenced by changing conditions of the internal as well as the external environment.

The amphibian chromosomes are cytologically of the hermaphrodite rather than of the gonochorist type. Sex-chromosomes of the *XO* or the distinctly heteromorphic *XY* type have never been found. This is the more significant, as amphibian chromosomes have been studied very extensively by many cytologists. Our

own investigations on *Rana temporaria* (Witschi, 1924, 1925b) show that females as well as males have a diploid number of 26 chromosomes. They can be arranged in 13 pairs of nearly even mates. It has been ascertained that the haploid number of 13 is characteristic for every gamete.

Since the existence of an *XY* pair is genetically established, special attention must be directed toward the critical phases of spermatogenesis. Heteropycnosis¹ of the sex-chromosomes in the digametic sex is a widespread feature among gonochorists. However, in the spermatocytes of *R. temporaria* all chromosome pairs behave in the autosomal manner: they expand into long fine threads, conjugate, then contract into ring or double-rod tetrads. The absence of heteropycnosis of the *XY* pair stands out as a feature characteristic of the hermaphrodite type. As first described in 1922, the fourth chromosome pair exhibits some peculiarities during the meiotic divisions that seem to mark it as the *XY* pair. In the first division its components move ahead of the other chromosomes to the poles, while in the second they lag behind. The two components of the first division are bipartite though equal; those of the second appeared to me slightly but distinctly different (Witschi, 1924). On rechecking my camera lucida drawings of spermatogonial mitoses it appears also that the fourth pairs as a rule show greater differences in length than any other. Makino (1932) and Galgano (1933) have reinvestigated the same species. Both have found again and illustrated the peculiar fourth chromosome. Makino also considers it as a sex-chromosome while Galgano denies it that title. Galgano's main argument, that the same features are also found irregularly in other chromosome pairs, proves only that his technique is not adequate to the task. Neither Makino nor Galgano has made an extensive study of the second maturation division. Their short statements of failure to recognise an *XY* pair are therefore of little weight. Future studies will have to concentrate on the second meiotic division, the measurement of spermatogonial chromosomes and the comparative analysis of spermatogenesis in sex-reversed females. For the present I still believe in the *XY* interpretation of the fourth chromosome pair. The circumstance that its cytological nature resembles more the autosome than the sex-chromosome type is highly in favour of my contention that the sex-chromosomes have arisen from an autosomal pair and that the frogs represent a very early phase of the evolution of the sex-chromosome mechanism.

My assumption of an *XY* pair rests primarily on the results of genetical analysis. Evidence of the same type has served to establish the *XY* condition in *Lebistes* and *Aplocheilus*. Even in *Lymantria*, in man, in the chicken and other cases, our knowledge about sex-chromosomes is based on genetical much more than on cytological facts. It is therefore a widely accepted practice to use the terms *X* and *Y* not merely in the cytological sense. Failure to find morphological differences cannot justify Iriki's and Makino's assumption of an *XX* condition in male frogs.

Levy (1915) had described an *XO* condition for *Rana esculenta*. We have always doubted the correctness of this statement, and Galgano's reinvestigation proves fully that it is erroneous and must be discarded. The *R. esculenta* male has 13 chromo-

¹ By *heteropycnosis* is designated the peculiarity shown by certain sex-chromosomes of maturing germ cells to become greatly condensed ("chromosome nucleolus"), while the other chromosomes preserve a thread-like or diffuse shape.

some pairs similar in shape and in number to those of *R. temporaria*. Beccari (1926) and Stohler (1926, 1928) found 11 pairs of chromosomes in both sexes of several species of toad, but no sex-chromosomes. Makino (1932), Iriki (1930) and Witschi (1933a), on the other hand, find that the fifth chromosome in *Bufo* shows peculiarities which bear out its homology with the supposed sex-chromosome of *Rana temporaria*.

No data are on record that would indicate the occurrence of morphological sex-chromosomes in urodeles.

While through genetical experiments it has been shown that in *R. temporaria* and *R. esculenta* the male sex is digametic, no similar evidence is available yet for any other amphibian species. Experiments of Harms (1926) and Ponse (1931) with sex-reversed toad males have not yet given conclusive information on the genetical constitution. Therefore we have to consider the possibility that some amphibians may be heterozygous in the female sex. Further studies in this interesting field are necessary. Considering the facts known at present we are inclined to predict that in most, possibly in all, amphibians the Y-chromosome will be found not an empty but a functional chromosome with cytological qualities similar to those of the autosomes.

IV. THE INDUCTORS.

All that we know about the properties of genes is inferred from reactions which they control during the course of development. If we say, for instance, that in hermaphrodites and intersexes the male and female genes are in a state of equilibrium ($\mathbf{M} = \mathbf{F}$), we refer not to the weight of the genes, or to the number of sub-genes or the volume of morphogenic substances released, but merely to the quantitative state that results in male and female differentiations of equal importance. In other words, we characterise the genes by their ultimate reactions. This is the usual practice in genetics. We may come closer to an understanding of their true nature, however, through the study of genic action during embryogenesis.

While engaged in my first investigations on sex differentiation in frogs (Witschi, 1914) I became aware that the sexual differentiation of the germ cells is induced from outside. Without regard to their genetical constitution, germ cells differentiate into oocytes if under the influence of the cortex, or into spermatocytes if under the influence of the medulla of the sex gland. Progressive work made it more and more clear that cortex and medulla play the rôle of inductors of sexual differentiation (Fig. 2b). We must conclude, therefore, that the \mathbf{F} genes act through the development of the cortical inductor and the \mathbf{M} genes through the development of the medullary inductor. In amphibians (and all higher vertebrates) both inductors are present in the embryonic gonad, and in many instances there is clear evidence that both are actively engaged in attempts to direct sexual differentiation. A most instructive case is that of the larval ovary of the toad (Fig. 2c). In its caudal parts the medullary cords (1-5) are active enough to induce the formation of abortive sex cords (S)¹ and spermatogonia. In the anterior part, on the other hand, the cortical

¹ *Medullary cords* are derived from the blastema of the mesonephric cords. They give origin to the urogenital connections and take part in the formation of the seminal tubules. *Sex cords* are strands of somatic cells and primordial germ cells growing from the cortex toward the medulla. They are most characteristic of the early phase of male differentiation (cf. Fig. 2c, which shows both types of cords).

influence is leading, as evidenced by the formation of rapidly growing young eggs. This case further shows that the difference between the anterior and the posterior region is related to the distribution of the medullary cords. A medullary cord distinctly retards differentiation and growth of oocytes in the nearest parts of the cortex, thus counteracting the cortical inductor. It is probable that it is a complementary effect of all embryonic induction to inhibit competing inductors.

It is a peculiarity of gonad development in the toad that no medullary cords are formed in the anterior half of the embryonic rudiment (Witschi, 1933*b*). Consequently, in the male, only the posterior half differentiates into testis. The anterior half remains a purely cortical organ of ovarian character, generally known under the name of "Bidder's organ." Thus a purely topographical factor causes the formation of a hermaphrodite gland despite the fact that the genetical situation is the same throughout the whole length of the gonad. The mere absence of the medullary cords in the anterior region prevents the possibility of action and expression of the **M** factors. The **F** factors, on the other hand, can go into action, since there is nothing

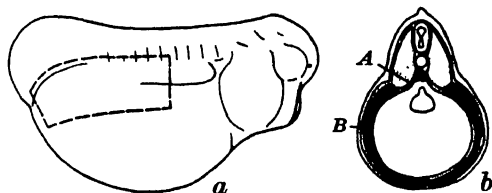


Fig. 3. Diagrams illustrating Humphrey's orthotopic transplantation of the presumptive somatic constituents of the gonads in embryos of *Rana sylvatica*. The area exchanged lies inside the broken line of *a* and between *A-B* of *b*. The latter figure shows also the primordial germ cells in the dorsal ridge of the endoderm, which in this experiment are not exchanged. (After Humphrey, 1933*a*.)

opposed to cortical development. These facts serve to illustrate our conclusion that there is no direct interaction, and especially no antagonism, between the **F** and **M** genes.

A series of embryological experiments furnishes more detailed information about the inductive process.

(1) It is well known that in frogs of the tailbud stage (Fig. 3*a*) the primordial germ cells form a dorsal crest above the roof of the posterior half of the primitive gut (Fig. 3*b*). Thus they lie well separated from the mesodermal nephrotomes and upper lateral plates out of which the inductor systems are to develop. We have dissected out this strand of primordial germ cells and implanted it into the anterior body cavity of some sister larvae, the development of which had been sped up somewhat by higher temperatures. The hosts were killed some time after their gonads had become sexually differentiated. The implants were found in the anterior body cavity, attached to the liver, the pericardium, or the body wall. In every instance they consisted of a large number of undifferentiated gonia, each surrounded by follicular cells. No attempt at gonad formation or sex differentiation has ever been observed. The presence of germ cells in ectopic¹ positions obviously does not cause

¹ Ectopic means out of its normal place.

the neighbouring mesoderm to form cortical or medullary inductors, nor are the germ cells capable of sex differentiation in the absence of these inductors. The capacity to form cortical and medullary inductors seems restricted to limited parts of the mesoderm in the mesonephric area. This conclusion bears on embryos just past the tailbud stage. About the distribution of this potentiality at earlier stages we have no knowledge as yet.

(2) An interesting experiment has been reported recently by Humphrey (1933*a*). At the tailbud stage he removes the so-called "intermediate mesoderm" (Fig. 3) of a host embryo and replaces it by the corresponding area from a donor of the same age. In accordance with chance expectation host and donor are of opposite sex in about half the cases (in fact, 13 out of 29). The strips of intermediate mesoderm contain the prospective cortical and medullary inductors. Therefore, in the hosts with heterosexual implants the inductor tissue of one side is of the **MMFF** and that of the other side of the **MMFf** constitution. On the contrary, the germ cells, still in the dorsal ridge of the endoderm at the time of the operation, are all of identical constitution, that of the host. Humphrey found that hosts with heterosexual implants developed into lateral hermaphrodites, the gonad of the implant side always assuming the sex of the donor. This experiment gives additional evidence for the above-mentioned fact that the germ cells have only a passive rôle in sex differentiation and become spermatogonia or ovogonia according to the type of induction to which they happen to be exposed. Humphrey's experiment is convincing to all who, like myself (Witschi, 1914*a*, 1929*a*), believe in the importance of the primordial gonia as the exclusive source for later germ-cell generations. This doctrine has its opponents and its sceptics, however, and among the latter has been found, so far, Humphrey himself (1927, p. 387; 1928, p. 88). Admission of secondary germ-cell formation out of mesodermal material obviously would delete the crucial importance of the experiment.

(3) The power of inductors is largely determined by their genetic constitution, though they are also quite sensitive to environmental conditions. In an experiment with *Rana sylvatica* (Witschi, 1929*b*) it has been observed that the cortical inductor in larval females is destroyed by a high temperature (32° C.), which still leaves the medullary inductor unharmed. After two weeks of "heat" treatment (temperatures above 32° C. have a lethal effect on this species), the females show characteristically modified ovaries. The germinal epithelium still contains some primary oogonia. Since the formation of new oocytes has been discontinued, early maturation stages are entirely missing. The young eggs of the auxocyte stage have disappeared or are in process of degeneration. It is evident that the cortical inductor has been put out of function. At this same stage now there follows a most remarkable reactivation of the medulla. In control females the medulla consists mainly of the epithelia of about half a dozen ovarian sacs. These epithelia in the experimental animals presently start a very copious growth, and within about two more weeks transform into massive racemose medullary cords. As in the normal development of the testes, these cords attract the primary germ cells which had remained in the cortex, giving thereby clear evidence of active function of the medullary inductor. The final result

of this experiment is the complete sex reversal of the females. Piquet (1930) has obtained a similar temperature effect in her work with *Rana temporaria* and *Bufo vulgaris*.

(4) The reverse experiment, keeping the developing frogs at low temperatures, gives also very striking though not as lasting results. It has been mentioned above that the Alpine races of *Rana temporaria* show a clear differentiation into 50 per cent. females and 50 per cent. males at an early larval stage. We have now to restrict this statement, since it holds true only for cultures that are kept between 15 and 25° C. As described above, higher temperatures cause the females to transform into males. On the other hand, if kept from the beginning at 10–11° C. the **MMFf** as well as the **MMFF** larvae develop in the female sense at the time when sex differentiation is due. Throughout the larval period morphological examination shows that all animals possess ovaries, and only when metamorphosis approaches can one note in some specimens that the medulla becomes more active. During and immediately after metamorphosis half of the animals (the **MMFf** group) transform into males. So far it has not been possible in frogs to suppress permanently the testicular differentiation of genetical males. Low temperature simply delays the activation of the male differentiator.

This, and the previous experiment, make it clear that the quantitative formula **FF** > **MM** > **Ff** expresses only the conditions found under average and nearly optimal temperatures.

(5) The cortex may be relieved of the pressure of the medulla more completely in the males of toads. As described above, one-third or even as much as one-half of the bufonid gonad is void of medullary cords. After surgical ablation of the testicular part the purely cortical Bidder's organ alone is left. All gonadal growth after such an operation consequently is cortical and therefore ovarian in type. It has been known, since the experiments of Harms (1921) and Guyénot and Ponce (1923), that this cortical lobe may grow until it attains the size of a normal ovary and produces mature and fertilisable eggs¹. Inasmuch as the male toad following this partial gonadectomy transforms into an egg-laying female it presents the aspect of experimental sex reversal. Embryologically, however, the ovarian lobe had differentiated before the surgical intervention; the latter merely occasions its growth to functional dimensions. The outstanding feature of this experiment is the demonstration that a specimen with the male genetic constitution develops as a female if the medullary inductors are completely removed while parts of the cortical inductors remain at the gonad site.

(6) The preceding set of experiments would be open to adverse interpretations had it not been shown that sex reversal is not accompanied by rearrangements in the chromosomal or genetical constitution. Microscopic examination of hermaphrodite frogs (Witschi, 1925*b*) and toads (Stohler, 1928) reveals the presence of the same chromosome equipment in male and in female germ cells. Moreover, the above-mentioned breeding experiments of Crew and Witschi have proved that the

¹ The same resumption of growth of Bidder's organ is observed in female toads after removal of the posterior, ovarian part of the gonad.

sex-reversed female frogs produce only gynosperms, which indicates that the spermatogonia of the ovotestes have retained the female (MMFF) genetical constitution.

The general conclusion to be drawn from the reported experimental facts is that sex differentiation depends upon inductors which in themselves are far removed from the primary action of the sex genes.

Another set of experiments provides us with more information about the interesting cortico-medullary antagonism.

(7) Embryos of the Californian newt, *Triturus torosus*, were grafted together while still at the tailbud stage. Some were united side by side into so-called parabiotic pairs, while in other cases an embryo was grafted on to the tailbud of another one (Fig. 4c represents both operations in a single diagram). According to the rules of chance combination one has to assume that genetical males and females become united in the ratio of 1 ♀♀ : 2 ♀♂ : 1 ♂♂. In the experiments there were actually obtained 35 ♀♀ + 69 ♀♂ + 36 ♂♂ (mostly unpublished data; for preliminary notes see Witschi and McCurdy, 1929; Witschi, 1930b). In the larval ♀♂ combinations one finds the testicular development distinctly retarded and the ovarian differentiation almost completely suppressed. In older specimens the testis recovers and becomes normal, while the ovary retains its rudimentary character or becomes even more degenerate. The various observations indicate that shortly before the time of morphological sex differentiation the gonads of both sexes start to emit hormone-like morphogenic substances, each of which tends to inhibit the development of the opposite sex. Since cortex and medulla are the inductive centres, the terms "cortixin" and "medullarin" have been introduced for the respective substances released¹.

There are only quantitative differences between twins and chains. In the latter the testes sometimes show no depression at all and the ovaries as a whole attain a higher degree of differentiation than in twins. In a study on the exchange of hypophysis hormones in parabiosis (Witschi, 1931b) it has been found that in twins often very large blood vessels anastomose, while in chains connections are established only by arterioles and capillaries. Correspondingly, hormones are more rapidly and more evenly distributed in the former. The fact that the antagonism between male and female gonads appears more severe in twins than in chains can therefore be considered as supporting evidence for the distribution of cortixin and medullarin by the blood stream.

(8) The mere presence of Bidder's organ, that is, of an ovarian lobe at the top of the testis, indicates that in toads conditions are somewhat different. The inhibitive power of each inductor evidently is short ranged. This has already been indicated by the conditions in the larval ovary. A closer study of Fig. 2c shows clearly the rapid falling off in the antagonistic effect with the increase of distance between inductive medulla and responding cortex. In parabiotic twins and chains of toads the

¹ Similar results, though with some unexplained variations, were obtained by Humphrey (1929, 1931b-d) with his method of orthotopic transplantation of gonadic preprimordia in salamanders. Elsewhere I have pointed out (Witschi, 1933c) that this method is objectionable because it provides no assurance against the mixture of all essential components of host and donor primordia on the operated as well as on the unoperated sides.

heterosexual pairs possess absolutely normal ovaries and testes (Witschi, 1933*b*). In other words, induced effects appear only in cells and tissues that are located near the inductor. Therefore, the morphogenetic substances in the toads are not emitted into the blood stream, nor are they evenly distributed over the whole system, but evidently they spread by a process of diffusion through the tissues (Fig. 4*a*).

(9) Frogs (*Rana* and *Hyla*) exhibit still a different situation in the transmission of the inductive stimuli. As in the newts, effects may be produced beyond the limits of the gonad that releases the morphogenic substances, though as in the toads we note a falling off in the effectiveness of induction with the distance from the emitting inductor (Witschi, 1931*a*). In parabiotic chains no antagonism between heterosexual gonads is ever observed, and in twins a distinct effect appears only if the animals are relatively closely united. In the latter case the inner gonads are sooner and more deeply affected than the outer ones (Fig. 4*b*).



Fig. 4. Diagrams to show the spreading of male induction in parabiotic combinations. *a*, toads; *b*, frogs; *c*, newts and salamanders. (After Witschi, 1932.)

Another striking difference between the parabiosis effects in newts and in frogs should be mentioned here. In the former the ovary, whose cortex becomes depleted by the antagonistic action of medullarin from the co-twin, remains in a rudimentary condition. Only feeble attempts at sex reversal are made, which never proceed beyond the very first steps of testicular development. In frogs, on the contrary, cortical inhibition is soon followed by the growth and testicular differentiation of the medulla. In other words, in frogs heterosexual parabiosis leads to sex reversal in the female twin. A comparison with the high-temperature experiment (No. 3) shows a close similarity in the process of sex reversal in both cases. We are, therefore, not inclined to believe that positive induction of testis differentiation goes from the male to the female twin. For, as in the case of parabiotic newts, it is sufficient to assume that merely the negative, cortex-inhibiting factor passes into the female twin. The testicular transformation follows as an autonomous and compensatory reaction after the deletion of cortical control. Why the same compensation does not follow under analogous experimental conditions in newts is not sufficiently clear. Indications are that the testes of the male twin check the development of new testes in the parabiont—not by sexual antagonism, but by a

process similar in nature to that by which the left ovary of the chick checks the further development of the right gonad.

The effects in the female member of a heterosexual pair of frogs appear then in the following sequence: (1) ovary with cortical inhibition, (2) hermaphrodite gonad, (3) testicular condition. This makes it possible to trace the gradual progress of induction as illustrated by the two cases represented in Fig. 5. In these and in other cases the inner gonads of twins have been found connected at their posterior ends. If they are of opposite sex, the testis always causes the partial or the complete sex reversal of the attached female gonad. As one would expect, this transformation progresses in a caudo-cranial direction. The present cases are of particular interest because in the non-attached gonad of the genetical females we find clear evidence of *secondary induction* issuing from the sex-reversed inner gonad. The outer gonad is most radically transformed where it comes near the posterior part of the inner gonad. From this area sex reversal progresses in both the cranial and the caudal direction

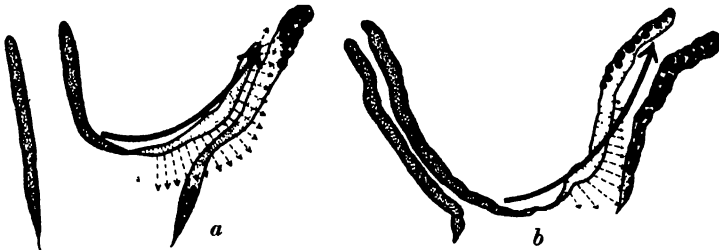


Fig. 5. Primary and secondary medullary induction in two pairs of parabiotic twins of *Rana aurora* in which the inner gonads are connected at their posterior ends. In both cases the right animal (left in the figures) is a genetical male and the left animal (right in the figures) is a genetical female. In case *a* the inner gonad of the genetical female is purely testicular; the outer gonad is ovarian on both ends and hermaphrodite or testicular in the central parts. In case *b* the inner gonad of the genetical female is testicular in its posterior half and hermaphrodite in the anterior regions; the outer gonad is an underdeveloped ovary with hermaphrodite features at the posterior end.

Dark arrows indicate the direction in which primary induction must have travelled from the testes of the male into the gonads of the female. Light arrows indicate the spreading of secondary induction from the sex-transformed inner gonad toward the outer gonad of the genetical female.

The testes of the genetical male are darkly stippled. Testicular and hermaphrodite parts of the genetical female are lightly stippled. Ovarial parts are marked with large dots. $\times 16$. (After Witschi, 1931 a.)

(Fig. 5a). Cases of this type prove clearly that the effect depends upon the concentration of medullarin to which each region of the gonad is exposed.

(10) In *Amblystoma maculatum* inherited sexual differences are found which make it possible to distinguish differentiated and undifferentiated races in a way similar to that in the above-described case of *Rana temporaria*. At the time of metamorphosis the local race of New Haven, Connecticut, consists of males and females without hermaphrodite features, and therefore it belongs to the first type. In the Ozark race (from Arkansas), on the contrary, the males of the same stage regularly possess a considerable vestige of ovarian cortex covering the crest of the testis. This race, therefore, belongs to the undifferentiated class. Embryos of the tailbud stage were grafted together to compare by the parabiosis method the

antagonism of cortical and medullary inductors of the two races. As in the previous experiments we need consider the heterosexual pairs only. They show significant racial differences. In pairs of the New Haven strain the gonads of the females suffer inhibitions nearly as severe as those described for the Californian newt. Oocytes are only rarely formed and the differentiation of ovarian sacs is completely suppressed in all typical cases. On the other hand, if the pair is of the Ozark race, the ovaries show only a slight inhibition in the growth of the ovarian sacs, while oocyte formation proceeds in the normal way. The results of these racial studies (Witschi, 1933c) are corroborated by Humphrey's most recent experiments on orthotopic transplantations (Humphrey, 1933b), and this investigator agrees with our conclusion that sex antagonism is low in forms which constitutionally are inclined toward hermaphroditism. The fact that the inductive antagonism is so much stronger in the

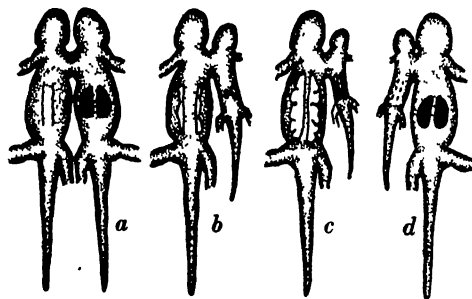


Fig. 6. Diagrammatic representation of sex conditions in heterosexual parabiotic twins of salamanders. The left animal always represents the genetical female, the right one the genetical male. *a*, any homogeneous pair or combination of two species of nearly equal size. Male (right) with normal testes. Gonads of the female (left) very reduced. *b*, *tigrinum-maculatum*; the *tigrinum* (left) ovaries more or less retarded. The *maculatum* (right) testes sometimes capped by ovarian cortex. *c*, *tigrinum-jeffersonianum*; the ovaries of *tigrinum* (left) are normal. The *jeffersonianum* twin (right), though genetically male, has no testes but atypical ovaries. *d*, *maculatum-tigrinum* or *jeffersonianum-tigrinum*. The gonads of the small twin extremely reduced.

differentiated than in the undifferentiated race is a significant guide to the understanding of the evolution of gonochorism.

(11) Heterogeneous parabiotic pairs, obtained by grafting together embryos of different species, give further evidence of the importance of the quantitative factor in the induction of sex differentiation. In combinations of *Amblystoma tigrinum* with *A. maculatum* the former is estimated to be at least ten times the weight of the latter at the time of metamorphosis. At the same time the volume of the testes shows about the ratio of 30 : 1. The testes of *A. tigrinum* not only grow faster, but they also proceed more quickly toward maturity than those of any other urodele so far studied. Accordingly, we find in the combination *tigrinum* ♂ + *maculatum* ♀ (Fig. 6d) the gonads of the latter extremely reduced (even more radically so than in *maculatum* ♂ + *maculatum* ♀ twins; Witschi, 1933c). In the reciprocal pairs *maculatum* ♂ + *tigrinum* ♀ (Fig. 6b) the *tigrinum* gonads always preserve an ovarian character with large eggs present in every case at the time of metamorphosis. The small *maculatum* male obviously is unable to suppress ovarian differentiation to the

extent that Burns (1930) observed in twins of *tigrinum* ♂ + *tigrinum* ♀ combinations (Fig. 6a). On the contrary, the *maculatum* male comes partly under the control of the *tigrinum* female. Its medullary development is in some cases suppressed to the extent that ovarian cortex of considerable size is permitted to develop (Fig. 6b, right gonad). Even smaller than *A. maculatum* is a local strain of *A. jeffersonianum* of Cedar Falls, Iowa. In combinations *tigrinum* ♂ + *jeffersonianum* ♀, the latter is always completely sterile (Fig. 6d). The combination *tigrinum* ♀ + *jeffersonianum* ♂ is phenotypically represented by double female pairs in which the *jeffersonianum* mate has rudimentary ovaries of an atypical shape (Fig. 6c). These "ovaries" are very slender tubes of mere cortex; the medullary cords are almost completely suppressed so that the sex gland is a morphological equivalent of Bidder's organ in the toad. Evidently, the disproportionate size of the parabionts results here in the reversion of the rule that in heterosexual parabiosis male dominates over female induction. In a forthcoming paper I shall give a more complete report on these heterogeneous twins of *Amblystoma*.

Striking differences in reciprocal combinations are also observed in *Amblystoma* + *Triturus* pairs. The testes of *Triturus* grow and mature extremely slowly, and accordingly a *Triturus* male interferes very little with the development of the ovaries of a co-twin *Amblystoma* female. In the reverse combination the *Amblystoma* male completely suppresses ovarian development in the co-twin *Triturus* female.

These heterogeneous combinations not only prove that the antagonism of inductors rests on a quantitative basis but they also demonstrate the non-specificity of the morphogenic substances, medullarin and cortixin, at least within the urodeles.

(12) In homogeneous twin pairs the effects of cortixin are not as conspicuous as those of medullarin. Yet we mentioned in Exp. 7 that in male-female pairs of the Californian newt the testicular, as well as the ovarian development, becomes inhibited during the early larval period. Later, the full dominance of the male is established. In a few cases of frog twins a temporary effect of cortical interference with testis development has been observed (Witschi, 1931a) which depends, however, on two contributing conditions: first, the two animals must be grafted together so as to bring their inner gonads close together (Fig. 7), and secondly, the male twin must be retarded in its early development. The latter condition is occasionally met with in any large experimental group. The case represented in Fig. 7 is especially remarkable, since it is the posterior end of the male gonad which lies nearest to the large ovaries of the female twin and which exhibits ovarian differentiation. Hermaphrodite gonads of frogs and of toads as a rule are more ovarian in anterior and more testicular in posterior parts. The sequence of this experimental case is, therefore, in opposition to the inherent tendency of the gonad which makes it even more certain that ovarian differentiation has been induced from outside. On the basis of previous experience we can infer that the cortixin of the female had spread into the male co-twin even before the onset of testicular differentiation, and that in the area of the posterior half of the inner gonad its concentration was high enough to suppress or retard the medullary development.

In frogs as in newts cortexin has the same mode of spreading from one twin into the other that is characteristic for the medullarin of the same species. In both groups male induction dominates over female induction if the twins are of identical species and identical developmental age.

The next three groups of experiments deal with the problem of sex reversal in the female-male direction, without, however, giving more than suggestive observations that may serve in the planning of new experiments.

(13) In 1910 and 1912 Meyns reported that in regenerating and in transplanted fragments of testicles of frogs he quite frequently found eggs inside as well as between the seminal tubules. Lauche (1915), repeating the same experiments with the same material in the same laboratory, obtained only male germ cells in his regenerates; he mentions, however, that he had seen the testicular eggs in Meyns' slides. Ponse (1924), working on testis grafting in *Bufo vulgaris*, claimed that every graft, homoplastic as well as heteroplastic, and no matter whether recovered from a subcutaneous or an intraperitoneal site, contains eggs. This testicular oogenesis is said to be at its height about eight months after implantation. Eggs do not surpass a diameter of 200 microns but finally degenerate. I mentioned one year later (Witschi, 1925*b*) that I was unable to find eggs in testicular implants, six months old, which were growing healthily under the skin of an adult female of *Bufo vulgaris*. Finally, in 1928, Welti, in an extensive paper on the subject, reported that eggs were found in 45 per cent. of recovered grafts in the same species. Deal (1929) and Uchida (1933), working again with frogs, found no eggs in the grafts, and equally negative were the experiments of Moszkowska (1932) with the fire toad, *Bombinator pachypus*.

Single eggs and groups of eggs are frequently found in normal testes of adult toads and not too rarely also in frogs. In young specimens belonging to sex races of undifferentiated type, eggs are often carried passively from the cortex into the medulla by the sex cords. However, not all of the testicular eggs are of this origin. In young males of toads the author has often observed germ cells, single or in small groups, in the lumen of testicular ampullae, that represented all stages of an atypical oogenesis from the undifferentiated primitive gonion up to testicular eggs of considerable size. Evidently the term "oviform degeneration" may justly be applied to this phenomenon. Oogenesis is atypical, since the synaptic phenomena (and apparently the whole process of chromosome conjugation) are omitted. The strands of chromatin of the primitive gonion change directly into the fine threads of the germinal vesicles. It is significant that this oviform degeneration takes place only in free cells with intact follicles. The follicles give origin to the fine granulosa which is the only envelope of testicular (intratubular) eggs. Naked germ cells are also frequently found floating in the lumen of seminal tubules, though they seem to degenerate quickly and without oviform transformations.

Essentially of the same type of oviform degeneration must be the formation of testicular eggs in grafts and in regenerating fragments. According to Welti (1928) the process starts in tubules with degenerating spermatogenic tissues. Within their canals one finds groups of abnormal germ cells which "seem to transform directly

into oocytes." The photomicrographs in the papers of Welti and of Ponse show clearly a fine follicular epithelium (granulosa) covering the intratubular eggs.

There is no doubt that these oviform transformations of germ cells within the testicular tubules are of the greatest importance for the development of the theory of induction. Their proper interpretation, however, is not an easy task in view of the diversity of the results reported and the incompleteness of the descriptions of the morphological process. Two significant features seem well established, namely, that the oviform process is observed with fair regularity exclusively in species (especially toads) with short-ranged medullary induction, and that oogenesis starts only after the testicular organisation has become weakened by extensive degeneration. Facts, therefore, indicate that the oviform germ cells have become freed from the control of the medullary inductor. This is not sufficient yet to explain the unmistakably

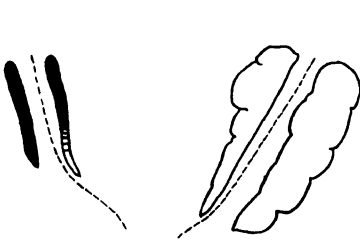


Fig. 7.

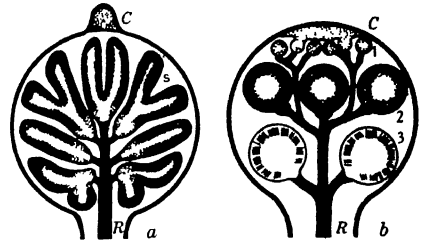


Fig. 8.

Fig. 7. Relative size and position of gonads in a pair of parabiotic twins (*Rana aurora*) in which the male differentiated later than the female. The posterior part of the inner gonad of the male is pre-ovulatory due to the inductive interference of the female twin. Testicular parts black, ovarian parts white. $\times 16$. (After Witschi, 1931a.)

Fig. 8. Persistence of cortical structures in testes of lower vertebrates. *a*, diagrammatic cross-section of testis of the Californian newt or of *Amblystoma tigrinum* at metamorphosis stage. Very commonly a vestige of cortex (C) persists at the crest of the testis; it is without connection with the seminal ampullae and disappears before attainment of the adult stage. *b*, diagrammatic cross-section of adult testis of a selachian, or of a European newt (*Triton*). A proliferating cortical crest persists throughout lifetime, giving origin to many successive generations of sex cords. C, cortex; R, rete testis; S, seminal ampullae (tubules); 1, 2, 3 successive stages of sex cords and seminal ampullae.

female character of the oviform degeneration. Since we came to the conclusion that sexual differentiation of germ cells is always induced from outside, we must try to locate the inductor also in the present case. It has long been my contention (Witschi, 1925b) that the follicle cells of the gonia (granulosa of the oocytes) form the component of the cortex which is responsible for its inductive actions. During testicular differentiation these follicle cells are carried by the sex cords into the medullary region, together with the germ cells. Under control of the medullary inductor they decrease or completely discontinue their output of corticosterone. In the light of this assumption the formation of testicular eggs in toads appears quite comprehensible.

(14) Beaumont (1929, 1932) in some instances found oviform transformations also in grafts and in regenerating fragments of testes of different European newts (*Triton*). Aron (1924, 1929), who has made extensive studies along the same line with the same species, never mentions the occurrence of eggs in his material. It is surprising that none of the students of the Geneva school has attempted an inter-

pretation of the egg formation on principles of the physiology of development. Beaumont, like Ponse and Welti, merely quotes Guyénot's genetical interpretation, according to which the egg formation should indicate a heterozygous sex constitution. The validity of their argument might well be doubted, since hermaphroditism proves merely the amphisexual, but not necessarily a heterozygous hereditary constitution. Moreover, the experimental facts demand an embryological rather than a genetical explanation.

The testis of *Triton*, as I can see from serial sections in my collection, has preserved an archaic type, otherwise unknown in adult tetrapods, though characteristic of selachians. In the latter a cortical germinal epithelium at the crest of the testis persists through the entire lifetime. It is the only store for residual gonia and therefore the permanent source of the successive generations of maturing male germ cells. During the reproductive period, sex cords continually carry clusters of germ cells into the medullary region where seminal ampullae are formed which make contacts with the branches of the rete cords (Fig. 8*b*). In *Triton* this cortical germinal epithelium also persists and in the adult continually gives rise to new sex cords and seminal tubules. Residual gonia, however, are found also in the older tubules. In the other tetrapods the sex cords exist only during the period of testicular differentiation. As a rule they convey the entire store of gonia from the cortex to the medulla within a very short time interval. In some urodeles (*Amblystoma tigrinum*, Burns, 1928; *Triturus torosus*, McCurdy, 1931) a number of gonia may be left behind at the crest of the cortex (Fig. 8*a*). They degenerate later, and at the adult stage the cortex of the typical amphibian testis consists only of the simple peritoneal epithelium. From this morphological description it appears that different zones of the *Triton* testis must be of different value. Small pieces from the region of the crest may consist mainly of cortex that becomes relieved of medullary control at the rate by which the deeper parts of the testis are eliminated. Beaumont's Fig. 24 (1929) clearly represents a case where the cortex under the described conditions produced a regenerate of ovarian character.

(15) We shall not discuss here the older work of Burns (1925) on parabiotic twins of *Amblystoma maculatum*. Reinvestigations of the same material by Witschi, Gilbert and Andrew (1931) and by Humphrey (1932), as well as Burns' own more recent experiments on *Amblystoma tigrinum*, prove that the conclusions arrived at can no longer be upheld. Nor can we attempt to analyse Humphrey's preliminary note (1933*b*) on interactions of ovary and testis in an Arkansas strain of *A. maculatum* because the published data are too incomplete. Several investigators, including myself, have used methods of implantation of gonads into the body cavities of larval amphibians to study the inductive antagonism. The results are in agreement with the parabiosis experiments, though the method does not give as regular and as clear-cut reactions.

V. DISCUSSION.

Inductors and inductive substances. The reported embryological experiments have led to the distinction of two inductors, *cortex* and *medulla*, producing inductive substances, *cortexin* and *medullarin*. We shall now proceed to reconsider some facts

that are most likely to lead to a clearer understanding of the nature of the inductive process. In frogs and toads the concentration of the inductive substances is found to fall off quickly with the distance from their source, while in salamanders and newts it appears nearly equal over the whole body, except that we must assume somewhat higher contents within the producing organs. These specific differences are diagrammatically represented by the curves of Fig. 9*a*. Comparing the effects in twins and in chains of newts (Exp. 7) we come to the conclusion that the zone of fusion is a partial barrier for the inductive substances. The smaller the anastomosing blood vessels the less inductive effect passes through (curve *Tt* in Fig. 9*b*). This relationship suggests that the inductive substances in newts are carried by the blood stream and therefore fall into the class of hormones. Salamanders, showing similar reactions, fit into the same scheme, though the rapid falling off of the effect in frogs and its strict and narrow limitation in toads show clearly that these substances are not always hormones in the classical sense. Since the physiological effects which they produce are so much alike, we must assume that the inductive substances are

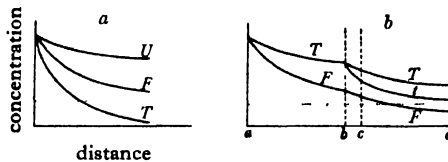


Fig. 9. *a*, diagrammatic curves illustrating the decrease in concentration of inductive substances with growing distance from the emitting inductor. *U*, salamanders and newts; *F*, frogs, *T*, toads. Dotted line indicates the threshold value. *b*, diagrammatic curves illustrating the decrease in concentration of inductive substances as they spread into parabionts. *a-b*, area of the emitting mate; *b-c*, zone of fusion; *c-d*, area of the receiving mate. Dotted line indicates the threshold value. *TT* represents the condition in parabiotic twins and *Tt* in parabiotic chains of the newt. *FF* illustrates the condition in parabiotic twins of frogs.

closely related, chemically, in the three groups. The fact that in frogs and toads they are not dissolved in the blood plasma but are moved and passed on through the tissues indicates differences in solubility. In this connection it is also significant that in heterogeneous combinations of frogs (*Rana sylvatica* + *R. temporaria*, or *R. sylvatica* + *R. pipiens*) no transmission of induction from one twin to the other has ever been observed, while, on the other hand, among urodeles induction is non-specific in the sense that it passes freely between twins of different species or genera (Exp. 11). We scarcely need to point out, after this, that the spreading of the inductive substances is in many ways dependent on local conditions and that, for instance, dispersion in the frog is not as regular as the diagrammatic rings in Fig. 4*b* might suggest. Body cavities and large blood vessels probably form barriers, and the tissues may have different conductive qualities. Irregularities of this type, however, do not invalidate the rules given for the three modes of distribution.

In this discussion we must also consider the fact that every inductor produces quite discrete stimulative and inhibitive effects. Stimulative induction causes the primordial germ cells to change into male or female gonidia. At the same time each inductor tends to suppress its antagonist by inhibitory operations. In parabiotic

twins we find frequent evidence that inhibition reaches over a much larger field than stimulation. In fact, all interactions between twins seem to arise from inhibitive induction only. Positive or stimulative induction appears narrowly confined within the sex glands and seems mainly to produce reactions by direct contact of inductor and receptor. Medullary stimulation also radiates across the albuginea to induce the formation of sex cords. In exceptional cases it causes, at the same distance, the transformation of young oocytes into giant spermatocytes (for instance, in the bull frog (Swingle, 1926)). Yet we do not know of any evidence of the spreading of stimulative induction beyond the gonads¹. Likewise, it is our impression that in cattle twins with fused foetal membranes inhibitors alone are exchanged, and that the attempts at testicular transformation, which are observed in some free-martin gonads, are merely a compensatory reaction following the destruction of the cortical inductor. The difference in range of stimulation and inhibition could possibly be explained on the basis of differential thresholds, though a better agreement with the observed facts is obtained by the assumption that the inductive substances are produced in pairs. Their respective components may then be designated as cortexin⁺, cortexin⁻, and medullarin⁺, medullarin⁻. Thus we conclude that within the taxonomic group of the amphibians the names "cortexin" and "medullarin" each stand for a whole series of inductive substances. Some of these have the character of hormones, being sent out by the inductor as messengers to distant parts of the body. Others spread slowly through the tissues with their effective concentration falling below the threshold value at some distance from the inductor.

One cannot fail to note the similarity of the inductors with Spemann's "organisers" of the amphibian gastrula. Since no blood circulation is established yet, we are not surprised to find that the inductive substances in the early embryos disperse in a way similar to that established in our work for frogs and toads (Fig. 4). Holtfreter (1933) has recently found that any part of the embryo outside the "organiser" (upper blastopore lip) will assume inductive functions after being killed by heat or by drying. Moreover, a multitude of tissues of embryonic and adult invertebrates and vertebrates produce distinct inductive effects if implanted in living or dead condition into amphibian gastrulae. The inductive power seems to be inherent in all animal tissues, and the problem arises how it becomes inactivated in the parts of the early gastrula outside of the "organiser" field in the blastopore lip. We believe with Spemann, Fischer and Wehmeier, and in opposition to Holtfreter, that here we have evidence of inhibitive induction. We intend to discuss this further in a separate study on accessory organiser formation in over-ripe eggs; for the present we merely emphasise the apparent parallel with the inductors of sex differentiation and their double function as stimulators and inhibitors.

Inductors of sexual differentiation are of a more specialised nature than the "organisers" of the blastopore lips. Organisers can appear in ectopic locations, such

¹ Here we consider only features of primary sex differentiation. It is well known, however, that secondary sex characters of any part of the body are stimulated by the activity of the endocrine glands of the gonads.

as the lateral or ventral sides of frog gastrulae. We have seen this happen in consequence of the disorganising effects of uterine over-ripeness on frog eggs (Witschi, 1922*b*, 1925*a*, 1934). On the other hand, inductors of sexual differentiation never arise outside the urogenital field (Exp. 1). Holtfreter's experiments at first sight seem to prove that the one inductive substance, whether coming from the organiser or from a piece of liver, stimulates differentiation of organs of all sorts and kinds (nerves, sense organs, limbs, gills, muscles, etc.). However, Mangold's (1933) observations on differential induction suggest rather that the "organiser" directly determines only a field of activity in which the more specialised inductors of actual differentiation presently appear according to patterns that have been little studied as yet.

The definite specificity of the cortical and medullary inductors in embryonic origin and functional reactions suggests again their likeness to endocrine glands. This resemblance becomes the more striking as facts accumulate to show that even true hormones are not always evenly distributed over the whole organism. In particular the hormones controlling secondary sexual characters have under certain circumstances a markedly lower concentration in peripheral regions (Witschi, 1933*b*). However, the inductors of sex differentiation are not identical with the endocrine glands of the adult gonads. The latter, as is well known, depress gonad development, and injection of the female sex hormone extracted from pregnancy urine has no influence whatsoever on the sexual differentiation of frog tadpoles (unpublished experiments).

Organisers, inductors and endocrine glands are closely related functionally as producers of morphogenic substances. They mainly differ in that they play their rôles at different developmental ages, assuming more specified functions at later embryonic and adult stages.

Inductive versus genic balance. Our quantitative formula $\mathbf{FF} > \mathbf{MM} > \mathbf{Ff}$ might create the wrong impression that sex determination is considered as the outcome of direct interaction between male- and female-determining genes. The embryological studies, however, disclose the fact that female and male genes independently determine the development of corresponding inductors. Antagonism makes its appearance only some time after these inductors have become established. This is most clearly brought out in parabiotic twins of different developmental age, where the earlier differentiating female temporarily dominates over the belated male (Exp. 12). That the quantitative ratio of the sex genes has no immediate influence on sex differentiation is demonstrated first by the many instances of hermaphroditism, where germ cells of identical constitution transform in the female sense if included in the cortex and in the male sense if in the medulla, and second by the sex reversal which follows immediately upon the experimental elimination of the leading inductor (Exps. 3 and 5). Parabiosis experiments with heterogeneous urodeles (Exp. 11) confirm the conclusion that it is the quantitative ratio of inductive substances that determines sex differentiation. It is true that under standardised developmental conditions the relative strengths of the inductors must depend upon corresponding quantitative ratios between the sex genes. This relationship permits

a quantitative comparison of these genes, though, obviously, there is no experimental evidence at hand to suggest interactions of any sort between the **M** and **F** genes directly.

These conclusions are in contrast with some of the ideas on genic action that Goldschmidt and Bridges have developed in connection with their work on *Lymantria* and *Drosophila*. We must consider, however, that in insects the embryological analysis of the chain reactions which lie between constitutional factors and final differentiations is especially difficult because, usually, the whole process is strictly localised, directing independent differentiation of smallest areas or even of single cells. The method of transplantation of inductors does not seem very promising in the field of insect embryology. However, the recent literature is full of indications that before long we shall obtain some insight also into the physiology of embryonic differentiation in insects.

Main factors and modifiers of genetic sex determination. It has been pointed out that in male toads an ovarian lobe—Bidder's organ—is regularly formed in those gonadal segments which are without medullary cords. The absence of these cords from the anterior segments is hereditarily fixed. While fundamentally a purely topographic factor, it assumes the character of a sex modifier. We could easily conceive of a genetical mechanism of sex determination based entirely on factors determining the relative locations of cortex and medulla. Jones (1932) has been successful in the construction of a self-perpetuating dioecious maize, based on two gene mutations ("tassel seed" and "silkliness") which certainly cannot be considered as anything but modifiers of the underlying monoecious sex condition.

Making a critical survey of the functions of our **F** and **M** genes we are struck by the close analogy with Jones' case of the maize. As the genetical mechanism of sex determination develops, neither better eggs nor better sperms are produced but hermaphroditism merely disappears. With increasing difference between **F** and **M** factors one, and alternately the other, sex becomes more efficiently suppressed. Comparing heterosexual twins of undifferentiated with those of differentiated races (Exp. 10) we note a striking increase in inductive antagonism between medulla and cortex, but no change in the morphological and physiological quality of female or male sex products. It seems that **F** and **M** genes acting through the cortical and medullary inductors simply determine what sex should be cut out, while the quality of the sex products depends on other factors which, at least in amphibians, are equally represented in the genetical constitution of males and females.

We know of only one group of evidence which may suggest that the **F** and **M** genes have a higher importance than merely that of gonochorism factors. Through the accumulation of genetical and cytological facts, of botanical as well as zoological origin, it becomes more and more clear that the establishment of the heterozygous condition in either the **M** or the **F** gene is followed by a series of characteristic changes in other genes. First, crossing-over decreases and finally disappears in the *Y*-chromosome, following the reduction and disappearance of the sex gene (**m** or **f**). Second, the reduction in the rate of crossing-over spreads over the autosomes of the heterozygous sex. Third, the *Y*-chromosome undergoes cytological changes of

which heteropycnosis and progressive size reduction are most characteristic. Conditions in coccids and aleurodids suggest that the hymenopteran type of sex determination may have evolved through degenerative elimination of a whole haploid set of chromosomes. It would represent, from this viewpoint, the ultimate consequence of the heterozygosity mechanism of sex determination. Fourth, genes residing in the *X*-chromosome together with the increasing **F** (or **M** in the case of female heterozygosity) acquire new potentialities by which viability of the heterozygous sex is preserved in spite of the gradual loss of the *Y*-chromosome.

The extent of these secondary changes and their analogy in different taxonomic groups creates the impression that **F** and **M** must be genes of fundamental importance: main factors of sex constitution. However, we do not know whether other genes, if kept perpetually in a heterozygous condition, would not produce similar effects. To decide this question it will not suffice to raise some hundred generations of heterozygous *Drosophila*; for time in evolution obviously is not measured in numbers of generations. Some information may be obtainable from the study of other cases of self-maintaining heterozygosity such as that of the heterostylic primroses. There is also some hope that this type of phylogenetic progress could be accelerated under laboratory conditions, since we have established that climatic factors are responsible for the differences between the sex races of *Rana temporaria*.

VI. SUMMARY.

1. Among amphibians, sex races have been found which are of particular interest because they exhibit different degrees of rudimentary hermaphroditism.
2. A genetical and cytological study of such sex races in frogs leads to the conclusion that they represent an evolutionary link between hermaphroditism and gonochorism¹.
3. The evolutionary trend from hermaphroditism to gonochorism, which is noted among different taxonomic groups of plants and animals, has an orthogenetic character and is evidently independent of selection. However, the special features of geographical distribution of the races of frogs (Fig. 1) suggest that certain climatic factors (temperature, duration of winter) may influence the speed of the evolutionary process.
4. The female frog is unigametic, the male digametic with respect to sex-determining factors. The genetic formulae are represented by **MMFF** = female, **MMFf** = male. The **M** (male) genes are probably located in autosomes, the **F** and **f** (female) genes in the sex-chromosomes *X* and *Y*.
5. Sex races differ chiefly in the quantitative values of the **f** genes, which are relatively high in races with pronounced hermaphrodite tendencies and low in races of more strictly gonochoristic character. The **F** genes vary also, but to a slighter degree. Their value is highest in races that come closest to the gonochoristic type. The factor **M** does not show any perceptible variation.

¹ See footnote 2, p. 460.

6. It is concluded that the genic or chromosomal mechanism of sex determination has been derived phylogenetically from the hermaphrodite condition, which may be expressed by the formula **MMFF** and in which **MM = FF**. In the development leading to male digamety, quantitative changes in the female genes accumulate, which leads to a diminution of the genes that remain mostly in the male line, and to an augmentation of those that are more often in the female line. Thus are established the *Y* (**f**) and *X* (**F**) chromosomes.

7. The condition found in frogs with female genes of low values in the *Y* and female genes of high value in the *X*-chromosomes is therefore considered as a transitional stage in the evolution toward the gonochorist type, in which **f = 0** and the *Y*-chromosome is empty or has entirely disappeared.

8. Self-fertilised eggs of adult hermaphrodites give, in addition to a mostly female offspring, also a few juvenile hermaphrodites. Their appearance may be explained by crossing-over between the two *X*-chromosomes of the parent, provided that the **F** factors are not one-point genes but consist of series of subgenes. However, further breeding experiments would be necessary to establish the subgene concept firmly.

9. Primordial germ cells, if transplanted to ectopic¹ regions, retain their undifferentiated character. Sexual differentiation occurs only if they become imbedded in cortex or medulla of gonads. With respect to this differentiation of germ cells the (somatic) cortex has the importance of a female inductor while the medulla functions as a male inductor.

10. Induction of sexual differentiation of the gonads is mainly by direct contact, though there are also cases of indirect transmission over short distances. It is therefore probable that the inductors produce their effects by the release of morphogenic substances; these are called *cortexin* and *medullarin*.

11. Besides this progressive or stimulative induction, the cortex, as well as the medulla, also show inhibitive functions by which each becomes the antagonist of the other.

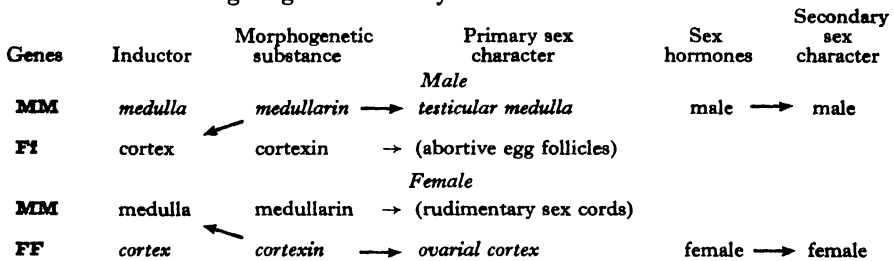
12. In genetical males the medullary inductor normally suppresses the cortical inductor. However, external factors (as low temperature, in frogs) more favourable to cortical than to medullary development can temporarily reverse the type of sexual differentiation. Surgical removal of the medulla—as in the partial castration of male toads—transforms a genetical male permanently into a phenotypical female.

13. In genetical females the cortical inductor normally suppresses medullary development. But external factors, such as heat or uterine over-ripeness of the eggs, may result in the selective destruction of the cortex, whereupon the genetical female develops as a phenotypical male.

14. It is obvious (from 12 and 13) that the female and male genes independently cause the appearance of the cortical and medullary inductors. In genetical males (**MMFF**) the cortex is weaker than the medulla and becomes suppressed in the normal development. In genetical females (**MMFF**) the cortex is stronger than the medulla and soon dominates the process of sex differentiation.

¹ See footnote on p. 470.

15. The relation of genes and inductors to sex differentiation can be represented in the following diagrammatic way:



16. Experiments in parabiosis show that the inhibitory action of inductors is narrowly confined in toads. It has a much wider range in frogs, though its effectiveness falls off with the distance from the inhibitor. In newts and salamanders it spreads over the whole twin system (Fig. 4). One must conclude from this that the morphogenetic substances which effect the inhibitions differ in solubility and in the way they spread. In newts they are like hormones, while in frogs and toads they spread through the tissues rather than by the blood stream.

17. Cortical and medullary inhibitions are of similar character, but in heterosexual pairs the male gains the lead in all cases of even-sized twins. In combinations of small males with large females the dominance of the male is incomplete or—in extreme cases—the female may assume the lead and bring about a peculiar type of sex reversal in the male (Fig. 6). It is thus evident that among the antagonistic cortical and medullary inductors the question of dominance and suppression is decided on a quantitative basis.

18. It is highly probable that each inductor produces separate stimulative and inhibitory substances which may be designated as cortixin⁺, cortixin⁻, and medullarin⁺, medullarin⁻.

19. The experimental data prove that the quantitative power of the inductors is primarily determined by the corresponding sex genes.

20. There is no interaction between male and female genes. The sexual antagonism, which is so characteristic for gonochorists, appears only between inductors, *i.e.* at a developmental phase quite remote from the gene.

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